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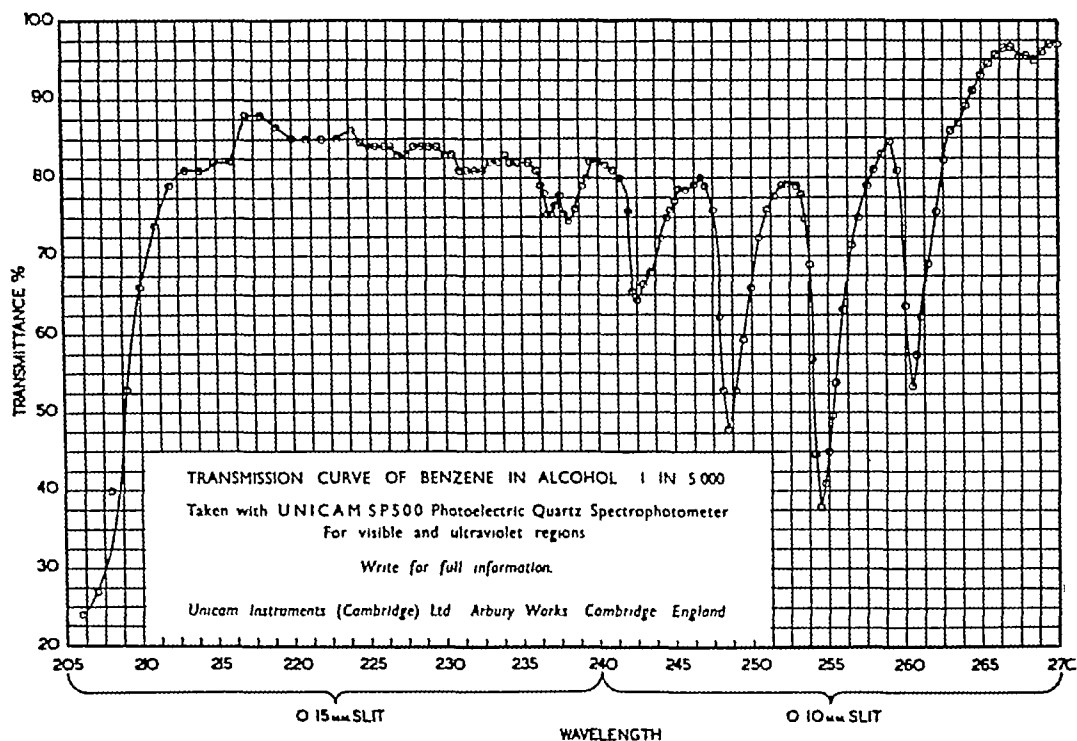
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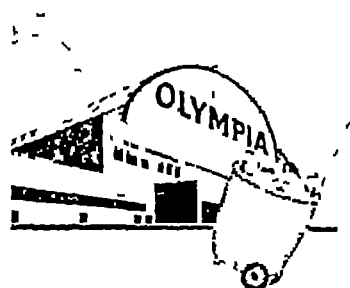
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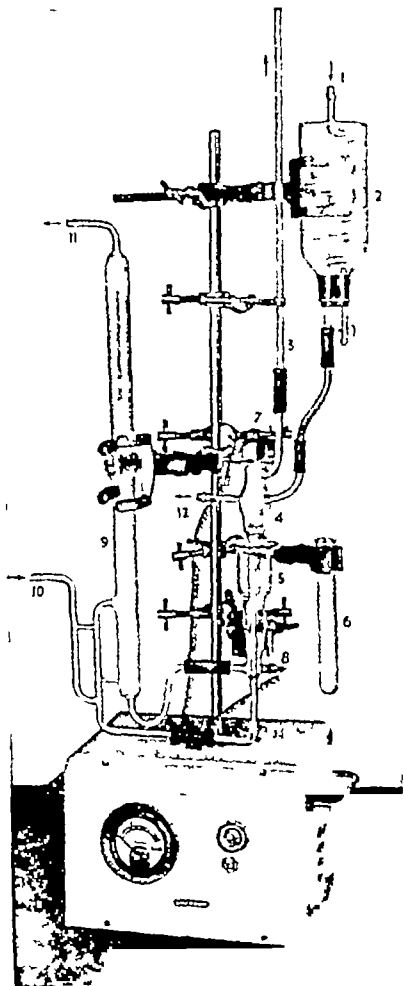
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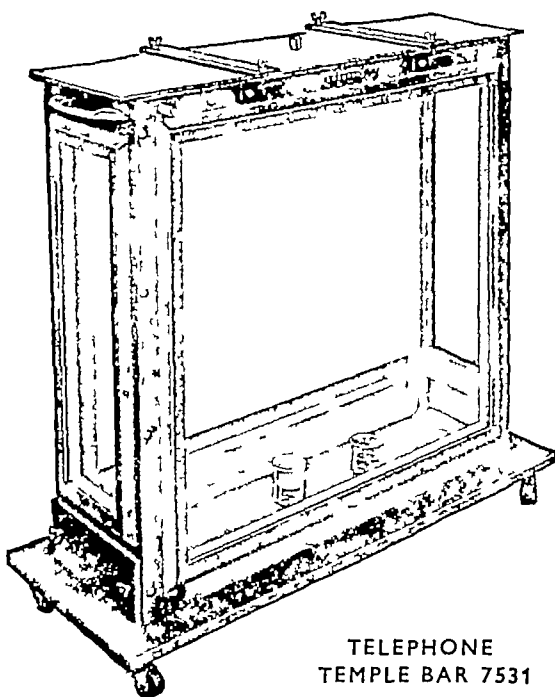
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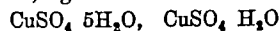
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The Potato Eelworm Hatching Factor

1 THE PREPARATION OF CONCENTRATES OF THE HATCHING FACTOR AND A METHOD OF BIOASSAY

B. C. T. CALAM, H. RAISTRICK AND A. R. TODD*

Biochemistry Department, London School of Hygiene and Tropical Medicine, and Chemistry Department, University of Manchester

(Received 8 April 1949)

The potato eelworm, *Heterodera rostochiensis* Wollenweber (Figs 1 and 4), is a strain or subspecies of the small nematode, *H. schachtii*, and until comparatively recently was generally described under the latter name. Eelworms parasitize a variety of economically important crops and are a serious menace to agriculture in many parts of the world. The parasite was first described by Schacht (1859), it was named *H. schachtii* by Schmidt (1871), who recognized it as a prevalent pest on sugar beet in Germany. In that country, after 1836 when sugar beet first became a farm crop, steadily increasing production without any regard to rotation led to a vast increase in eelworm infestation, with the result that between 1870 and 1880 crop failures became frequent. Only by suitable rotational cropping and intensive cultivation could the pest be brought under control. In England, Massee (1913) described the disease known as 'potato sickness', the symptoms being slow growth, spindly stems and unhealthy foliage, and noted that it seemed to be associated with the presence of eelworms in the soil. Increases in the disease and in eelworm population were rapid in the period of intensive potato cultivation during the 1914-18 war, and potato sickness has now become, in this country, one of the most serious and wide spread of potato diseases, having become even more prevalent during the recent world war. It should, perhaps, be mentioned that the severity of potato sickness may also be affected by unknown factors, as it cannot always be directly correlated with the population of potato eelworm cysts (cf. Buckhurst & Fryer, 1930; Ellenby, 1942).

Although *H. schachtii* had been reported as attacking, amongst other plants, beet, potatoes, oats and peas, it was observed that each strain became specialized to a single host and lost its ability to parasitize others. With this specialization came small changes in morphology, so that a subdivision of *H. schachtii* became necessary. The potato strain, characterized by the almost spherical shape of the mature cysts (Fig. 2) as distinct from the lemon

shaped cysts of the beet strain, was given the name *H. rostochiensis* by Wollenweber (1923).

The life history of *H. rostochiensis* is well known. The larvae hatched from cysts in the soil enter the growing potato plant after some 17 days by drilling a hole through the root wall. There the worms are true parasites until they attain maturity, when the males escape into the soil and the females remain attached to the roots by the head. The body of the fertilized female swells to a white roundish cyst full of undeveloped eggs, gradually becomes brown and then contains several hundred fully formed larvae (Figs 5 and 6), and drops off into the surrounding soil as a mature cyst. The cysts lie dormant in the soil until stimulated to hatch by the growing of a new crop of potatoes. They can remain dormant but viable for several years, and indeed cases have been reported where cysts were still in part viable after 10 years. The disastrous effect of continued cropping on the same soil is thus readily understood, as is the value of a rotational system with several years between successive potato crops.

The hatching of larvae from cysts of *H. rostochiensis* occurs in response to a specific stimulus due to a secretion from the roots of the host (Fig. 7). This appears to have been first realized by Baunacke (1922), in the absence of the stimulus the cysts remain dormant, and hardly any larvae hatch (Fig. 3). Baunacke tried the effect of various chemical substances as hatching agents and claimed that ferric chloride, potassium permanganate and bleaching powder had some effect. O'Brien & Prentice (1930) showed that potato eelworm cysts could be made to hatch with 'potato root excretion' but not with secretions from beet, rape, mustard or oats. Triffitt (1930) was the first worker to investigate 'potato root excretion'. This author showed that it was non-volatile and reasonably heat resistant, and that it retained its activity for some time in sterile water. Later, Hurst (1935, 1937) attempted to isolate the hatching factor from leachings of soil in which potatoes were growing. By evaporation and ethanol precipitation he obtained powders which brought about hatching at a limiting dilution of

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1/500,000. On very scanty evidence he formed the opinion that the factor was an amino acid and stated that several amino acids, in particular tyrosine, showed some activity.

The isolation and structural elucidation of the potato eelworm hatching factor are problems of considerable scientific interest, but might also have very important practical consequences for agriculture. Much work has been devoted to the control of the pest, but no really satisfactory means of eradication has been found. Various chemicals have been used to treat the soil, of these, calcium cyanamide and chloroacetate seem to have shown some promise, but are uneconomic. More recently, dichloropropane dichloropropane mixtures have been used to control, if not to eradicate, eelworm, such mixtures have been recommended in this country for the control of *H. marioni* in greenhouse plants (cf. Ministry of Agriculture and Fisheries, 1947). Recently, Ellenby (1945) has reported satisfactory results in experiments in which allyl isothiocyanate is applied to soil infested with eelworm, these findings, which parallel the earlier observations of Smedley (1939) with phenyl isothiocyanate, may explain the low incidence of eelworm attack on potatoes grown together with white mustard on infested soil (Morgan, 1925). The main difficulty lies, of course, in the dormancy of the cysts, for until the larvae emerge they are largely protected from attack by many chemical agents. This being so, an obvious alternative would be to apply a hatching factor to infested soil in the absence of potato or other plants capable of being parasitized by the eelworms, the emergent larvae could be allowed to die in the absence of a suitable host, or alternatively, would be readily open to attack by chemical agents. The natural hatching factor might well prove too complex for economic synthesis on the large scale needed if it were to be so used, but if its structure were known it might be hoped that some simpler related substance might have a similar action and be more easily prepared.

The investigations described in the present paper were initiated in 1939 at the London School of Hygiene and Tropical Medicine and at Winches Farm, St Albans, where the production of material was first undertaken with the help of Miss M. Oliver, they were transferred to the University of Manchester at the beginning of 1941 and continued there. At the outset it was clear that the methods employed by Hurst (1935, 1937) on potato root excretion were too cumbersome. Following a suggestion made by Prof. R. T. Leiper, whose assistance in the early stages of the work is gratefully acknowledged, it was decided to use the leachings from growing tomato plants instead of potatoes, since he had found that such leachings were highly active in hatching potato eelworms, moreover, tomatoes have the added

advantage that they are more conveniently grown and handled in bulk indoors. The method finally adopted for preparing crude solid concentrates of the hatching factor is described in detail below. Plants were grown in soil in pots or trays and watered frequently, the leachings being collected. From the leachings, active material was adsorbed on activated charcoal from which it was eluted with aqueous acetone, evaporation of the eluate under reduced pressure gave a brownish solid showing high activity when tested on potato eelworm cysts. The amounts of material obtained were not very large. For example, continuous cultivation of 4000-5000 plants yielded in the course of 1 year some 27 g. of the crude solid mentioned above, this solid had an average Relative Activity (R.A.) of 6.5 (see p. 518) and probably contained less than 2% of the factor itself.

EXPERIMENTAL

Isolation of crude material containing hatching factor

The preparation of hatching factor concentrates can be conveniently divided into four sections: (1) leaching, (2) adsorption, (3) elution and (4) evaporation.

(1) *Growing and leaching of tomato plants.* As production of fruit was not required the plants were kept quite small, constant over watering had in any case a bad effect on their development. Such experiments as were carried out showed that the most active leachings were obtained when the plants were 6-9 in. high with vigorously growing roots. It was thus necessary to keep a good supply of new plants coming along and to discard them after about 3 months, since by that time the yield of hatching factor had fallen off very markedly. Old plants could be 'reconditioned' by cutting off the tops and replanting the roots in fresh soil, but on the whole it was found better to discard them and use new seedlings.

The plants were established under the usual conditions. The seedlings were planted in 3 in. pots containing a suitable tomato growing compost. After about 6 weeks they were well developed and leaching was begun. To facilitate collection of the leachings the pots were kept on tables with sloping tops made of asbestos sheeting or of boards covered with rubber 'roofing felt'. The tables were usually about 8 ft. long with a slope of 3 in. to one end, and some 4 ft. 6 in. wide with a slope of 3 in. towards a central gutter to carry off the leachings to an enamelled container. A table of this size held 215 plants. Watering was carried out on alternate days using water at the same temperature as the greenhouse. The space of about 1 in. between the earth and the top of each pot was usually filled with water which was allowed to soak in and the process repeated once. From 4000-5000 plants treated in this way, the yield of leachings was about 100 l./day, giving about 500 mg. of crude active solid when worked up as described below.

(2) *Adsorption.* The leachings, which were light orange in colour and had pH about 6.5, were poured without filtration into vessels containing charcoal (British Drug Houses Ltd., 'Activated Charcoal for Decolorizing Purposes' was found most suitable), 1 g. charcoal being used/l. of leachings. The charcoal gradually settled out on standing, and after 1 day the supernatant liquid was siphoned off and discarded. The charcoal adsorbate was collected on a Buchner funnel and

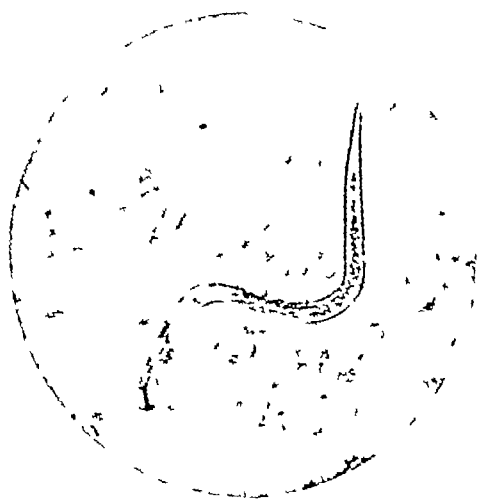


Fig 1 *Heterodera rostochiensis* The potato eelworm
Magnification, $\times 130$



Fig 2 Cysts of the potato eelworm
Magnification, $\times 30$

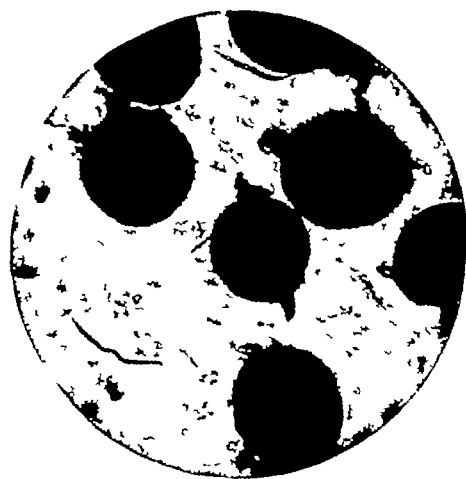


Fig 3 Cysts of the potato eelworm soaking in water,
two worms have hatched Magnification, $\times 30$



Fig 4 Potato eelworms hatched from
cysts Magnification, $\times 30$

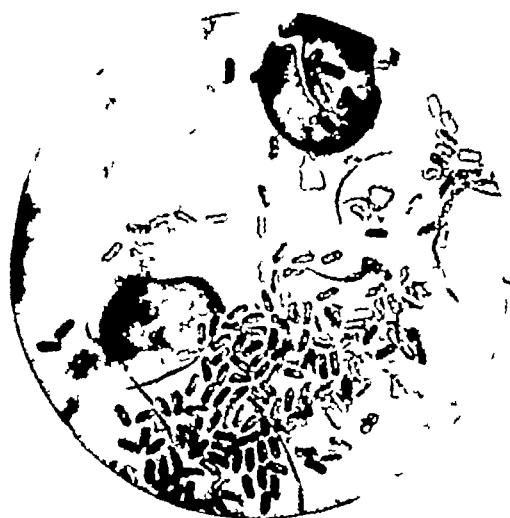


Fig 5 A dissected cyst of the potato eelworm, note the immature larvae and a few mature worms. Magnification, $\times 30$

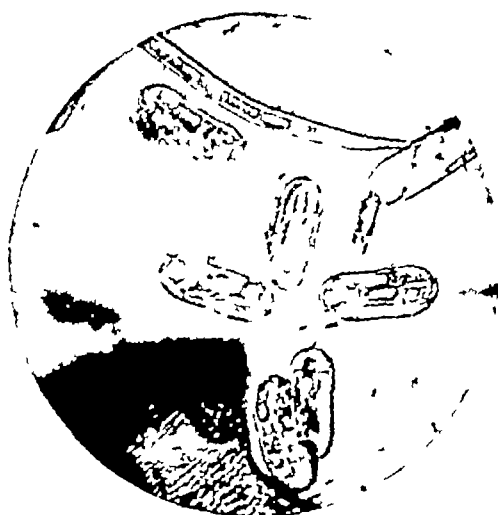


Fig 6 Immature larvae and part of a mature eelworm. The typical coiled form of the worm in the larva can be discerned. Magnification, $\times 130$



Fig 7 Cysts of the potato eelworm soaking in a solution of hatching factor. Twenty worms have hatched (cf Fig 3). Magnification $\times 30$

used as dry as possible. The loss of activity in this process is probably about 10%. Various other adsorbents were used and found inferior to charcoal.

(3) *Elution* The moist charcoal adsorbate was generally collected and eluted in 1.5 kg. batches by stirring with 8 l. of aqueous acetone (70% acetone) in four lots. The eluate was pale yellow, the charcoal recovered could not be used again and was discarded.

(4) *Evaporation* The filtered eluate was evaporated under pressure of 15 mm., the temperature being kept below 30° and the evaporation residue finally dried in a desiccator at room temperature over H₂SO₄. The product was a light brown powder, readily soluble in water, and contained some 50–70% of the active material in the leachings. It formed the starting material for all the investigations here described. During the year 1940, before the concentration procedure had been standardized, a total of 76 g. crude solid of average R.A. 1 was obtained from plants grown at Winches Farm, St Albans. During 1941, 27 g. solid of average R.A. 6.5 was obtained from the same source, together with 9.5 g. of average R.A. 6.2 from the Hawthorndale Laboratories of Imperial Chemical Industries Ltd., stages (1) and (2) in the concentration were carried out at these places and the charcoal adsorbate was then worked up during 1940 in London and subsequently at Manchester.

Preliminary studies on the crude hatching factor

The crude solid obtained as above appeared to consist largely of a mixture of salts (mainly calcium) of organic acids. As a preliminary to concentration studies, the stability of the factor at various pH values was examined. The results obtained are shown in Table 1. It is evident that the factor, although moderately stable at pH 1–7 (allowing for some latitude in the biological assay values), is extremely rapidly deactivated at pH values above 7, both at 100° and at room temperature. In practice, the active principle in the crude solid frequently appeared to be much less stable than the values in Table 1 would suggest, activity being all too often

other than very small quantity, has throughout seriously hampered chemical investigation.

As the active principle appeared to be acidic, various attempts were made to effect purification by precipitation as a metallic salt. These failed, the salts being uniformly too soluble in ethanol and their preparation being accompanied as a rule by marked loss of activity which was not restored on acidification. The factor was evidently of low molecular weight, since it diffused through a collodion membrane, and no marked concentration was obtained by electrodialysis. The crude solid gave no characteristic colour reactions, and, although it gave a reaction with carbonyl reagents, the active principle itself did not seem to be ketonic, since treatment of a solution with Brady's reagent and removal of the small precipitate of 2,4-dinitrophenylhydrazone had no marked effect on its activity.

BIOASSAY OF THE HATCHING FACTOR

Before serious chemical investigation of the potato eelworm hatching factor could be undertaken it was necessary to devise a reliable bioassay method. The literature revealed no method meeting our requirements, and comparatively little was known of the factors influencing the hatching of cysts. The usual method employed was to place a number of soaked cysts in dishes (e.g. deep solid watch glasses), add root excretion from potatoes and count the larvae produced. Hatching was usually allowed to continue for several weeks. Triffitt (1930) followed this procedure using, as a rule, single dishes at 25°. Franklin (1940) stressed the use of several dishes for each test in order to make possible a statistical examination of results, and drew attention to the unreliability of methods then available. Gemmell (1940) criticized this technique and preferred to study the hatching of single cysts. Using a method of his own, in which about 100 single cysts are treated, he examined cysts obtained from different potato growing areas and showed them to differ in their hatchability, although they were otherwise alike. In particular, he drew attention to the important fact that, although cysts may appear perfect and be full of embryonated eggs, on test a considerable number always fail to hatch. In Gemmell's experiments hatching was allowed to continue for some months.

For chemical tests, such methods as the above were too slow and unreliable. It was, therefore, decided to carry out a series of investigations aiming at a quantitative method for testing concentrates for hatching activity, which could be carried out in a few days at any time of the year. It seemed possible that this might be achieved, despite the seasonal variation in hatchability, reported by Triffitt (1930), if attention were paid to source and pretreatment of

Table 1. *Stability of hatching factor in crude solid under varying pH conditions*

pH	Half life at 100° (min.)	Half life at 25° (hr.)
1.1	6	Very great
1.8	9	"
2.7	15	"
3.6	15	"
4.8	8	"
5.8	17	"
6.8	8	"
8.1	2.5	1.5
9.1	1.5	—
10.4	0.25	0.15
11.2	0.25	—
12.1	—	0.0

lost for no apparent reason. This instability, coupled with the difficulty of accurate bioassay and the fact that the starting material was difficult to obtain in

eelworm cysts as well as to conditions of hatching. The cysts used were obtained from two sources, sample *A* from eelworm infested soil in Hertfordshire and sample *B* from similar soil in Chat Moss, Lancashire. The preparation of hatching factor used throughout the experiments, and described as 'Standard Solid', was a sample of crude solid material obtained in early experiments on tomato root excretion in which 5 g charcoal were used for each litre of leachings, and the aqueous acetone eluate was evaporated to dryness. Several grams of this product were set aside for use as a standard preparation, under suitable conditions it gave good hatches with viable cysts at dilutions from 1/20,000 to 1/40,000. Sample *A* cysts were on the average smaller and hatched more rapidly than those in sample *B*, although the latter were quite satisfactory to work with, and were otherwise very similar to those of sample *A* in behaviour. The specific objective of the experiments carried out was a simple test method, and not an exhaustive study of all the important factors in cyst hatching.

hatching. Excluding cysts which failed to hatch at all, larval counts varied from 1 to 195, while the standard deviation of individual results was considerably greater than the mean.

Sample B cysts These cysts were isolated by the usual flotation method from soil obtained from Chat Moss, Lancashire, through the kindness of Dr R Stewart and Dr V E Henderson of Manchester University. Collection was made in August 1941, from a field in which potatoes were growing (the fourth successive crop), so that the cysts were probably at least a year old. As they varied considerably in size, the cysts were graded into three groups by sieving, approximate numbers/sack of soil being (1) held by 40 mesh sieve, 22,000, (2) held by 60, passed by 40, mesh sieve, 60,000, (3) held by 100, passed by 60, mesh sieve, 60,000. The cysts obtained were stored at about 23° in a desiccator over saturated K₂CO₃ solution giving a relative humidity of some 50%. The intermediate group (2) was selected for general use and is described as sample *B*. As the technique of sorting previously used was rather tedious, the following method was adopted. The cysts were placed in a dish of water, stirred thoroughly and left for 2 days, after which time those that floated were rejected. Those which sank were sorted under

Table 2 *Hatching of single cysts from Sample A*

	Exp 1	Exp 2
	45 cysts treated with 1 ml Standard Solid, 1/40 000 in tap water, for 3 days at 24°	45 cysts treated with 1 ml Standard Solid, 1/20,000 in tap water, for 3 days at 24°
Hatches	11, 8, 0, 11, 10, 9, 27, 33, 1, 10, 0, 1, 0, 16, 3, 13, 0, 34, 7, 1, 0, 0, 0, 1, 0, 21, 0, 2, 0, 1, 7, 36, 1, 34, 46, 2, 4, 0, 1, 2, 1, 51, 0, 1, 0	13, 73, 63, 15, 0, 1, 0, 0, 12, 38, 1, 1, 19, 96, 4, 23, 0, 24, 41, 21, 46, 50, 24, 26, 16, 13, 1, 64, 1, 74, 25, 70, 0, 20, 195, 0, 1, 54, 0, 7, 34, 32, 0, 5
Total hatch	406	1203
s.d. among individual results	14	36
Mean hatch	9	27
s.e. of mean	±2.1	±5
Hatching no larvae (%)	20	20
Hatching only 1 larva (%)	20	13
s.e. of mean hatch on the basis of a count of 80 cysts	1.5 (=17% of mean hatch)	4 (=15% of mean hatch)

Sample A cysts The soil was supplied by the late Mr A. C. W. MacDonald of Winches Farm, St Albans, and was collected in the autumn so that most of the cysts were probably young. The cysts were isolated from the soil by a flotation method followed by rolling off on a Bristol board, as described by Fenwick (1940), and were stored at room temperature in a closed jar. When required for use, batches of cysts were soaked for 2-4 weeks in water at 24° and then sorted under a dissecting microscope by Miss M. Oliver. Viable cysts were selected by their appearance in conjunction with the results of dissections of small numbers of samples for this purpose, and were kept in water at 24° until used, except in experiments dealing with the effect of temperature of storage.

The hatching of single cysts was examined as described by Gemmell (1940), and the results of two experiments are given in Table 2.

From these results it is evident that not only did many apparently normal cysts fail to hatch, but there were also great variations in the rate of

a large lens in a strong light, when viable cysts could be readily distinguished by their uniform light brown colour and resistance to pressure with a blunt glass needle. The selected cysts were normally kept in water at 22° and used after about 3 weeks.

Table 3 *Hatching of single cysts from sample B*

(25 cysts treated with 1 ml Standard Solid, 1/30,000 in tap water, for 3 days at 23°)

Hatches	3, 0, 0, 15, 0, 7, 7, 7, 0, 4, 4, 4, 49, 5, 17, 0, 0, 0, 0, 0, 43, 5, 4, 0
Total hatch	174
s.d.	13
Mean hatch	7 ± 2.6
Hatching no larva (%)	44

On the basis of a count on 80 cysts, the s.e. of the mean would be 1.5, or ±21% of the mean hatch.

From Table 3 it is clear that the behaviour of sample *B* cysts is rather similar to that of sample *A*, and that the variable distribution of good and inert cysts among the dishes makes a high standard deviation inevitable

It was noticed in the course of sorting the Chat Moss cysts that in the case of the smaller cysts (groups 2 and 3) approximately one third appeared viable, whereas the proportion of good cysts in group 1 was much less and nearly 90 % were empty. It is of interest to note that a quantity of soil obtained from the same field 8 months later (after the potato crop had been harvested) was found to contain some 70 % more cysts/sack and there were virtually no large cysts present, the proportion of viable cysts was about the same as in sample *B*.

Earlier workers (e.g. Triffitt, 1930) have reported marked seasonal variation in ease of hatching. To check this, a portion of sample *B* cysts was set aside over saturated potassium carbonate under the roof tiles during the autumn and winter of 1941-2, when they were exposed to considerable variations in temperature, frost and snow being frequent. At intervals of about 1 month, samples were removed, sorted as above described and the viable ones soaked for 3 weeks at 22° and tested for hatchability by incubating in groups of 15 for 3 days at 23° with 1 ml of Standard Solid solution (1/40,000). Simultaneous tests were made on samples from a second batch stored continuously at 23°. In both batches it was observed that cysts hatched fairly well (about 150 larvae from 15 cysts) in August, but this gradually fell until, by the end of November, very low hatches (10-20 from 15 cysts) were obtained. Hatching remained very difficult throughout December and January, and began to improve again thereafter. Since it was shown by both batches of cysts, the variation in hatchability is evidently seasonal and not due simply to temperature of storage. In later work on the eelworm hatching factor we have always observed this effect and, indeed, great difficulty has been encountered in doing any reliable testing of concentrates during the winter months.

In the above experiments, cysts were usually soaked in water for at least 3 weeks before a hatching test was made. This practice was adopted as a result of experiments on the effect of soaking on hatching. A batch of viable sample *B* cysts was placed in water, and samples were removed and tested with Standard

Solid solution (1/30,000) from time to time. The results are given in Table 4. The actual hatches are low as the experiment was made in January when cysts are rather difficult to hatch, but the effect of soaking is evident.

Table 4 *Effect of soaking cysts*

Period of soaking (days)	Mean hatch/dish of 20 cysts (3 days at 23°)
2	0
8	1
15	12
18	12
21	71
23	53

The effect of temperature on hatching and on soaking was examined in other experiments. In one of these, sample *A* cysts were kept at 24° for some weeks and then incubated at various temperatures with Standard Solid (1/30,000). The results are shown in Table 5. In the experiment leading to the results in Table 6, sample *A* cysts were sorted after 3 days soaking, and batches were then kept soaking for 11 days at various temperatures before hatching in the usual manner. Although these experiments are only very rough (their extension, although desirable, was rather beyond the scope of our present researches), the importance of temperature emerges quite clearly.

Table 5 *Effect of temperature on hatching*

Temperature during hatching	Mean hatch/dish (15 cysts, 3 days)
6°	2
Room temp (about 15°)	16
24°	608
37°	7

Table 6 *Effect of temperature of soaking*

Temperature of soaking	Mean hatch/dish (15 cysts, 3 days at 24°)
6°	4
Room temp (about 15°)	270
24°	680

For setting up a rapid testing method experiments were carried out to determine the time required to give a suitable hatch and maximum reproducibility. The results of a typical experiment are summarized in Table 7.

Table 7 *Mean hatch per dish*

Dish no	Mean hatch per dish																Standard deviation	Mean hatch
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
Day 1	111	48	29	18	125	61	51	87	33	6	85	80	142	75	31	135	42	70 ± 10.5
2	239	230	160	70	316	236	251	341	250	75	255	275	325	315	145	350	85	233 ± 21
3	274	325	210	104	420	285	346	540	305	125	356	316	431	403	207	463	120	319 ± 30
4	340	263	206	119	415	287	300	552	333	162	385	377	425	365	227	570	127	332 ± 32

Table 8 *Calculation of standard error of means using different numbers of dishes*

Day	Using 6 dishes of 15 cysts	Using 4 dishes of 15 cysts
1	17.1 or $\pm 24\%$ of hatch	21 or $\pm 30\%$ of hatch
2	34.7 or $\pm 15\%$ " "	42.5 or $\pm 18\%$ " "
3	49 or $\pm 15\%$ " "	60 or $\pm 19\%$ " "
4	52 or $\pm 16\%$ " "	63.5 or $\pm 19\%$ " "

In this experiment 16 dishes were prepared each containing 15 cysts (sample A) to which 1 ml Standard Solid solution was added. The dishes were incubated at 23° and counts made after 1, 2, 3 and 4 days. Table 7 shows the hatches obtained in each dish together with the standard deviations of the means.

In Table 8 the results have been further subdivided to give standard deviations calculated for groups of six or four dishes. It will be seen that hatching had practically ceased by the third day and that the standard deviation had fallen to a steady proportion, about 10% for 16 dishes, by the second day. With six or four dishes the standard deviations were 15% or 18–19%. Although it was convenient to use conditions where the number of larvae were from 180 to 200/dish, Table 3 shows that the standard errors of means were similar when the counts were much lower. From the results given it will be seen that with three to four dishes containing a total of 45–100 cysts standard deviations of about 20% are to be expected. General experience confirms this. For the purpose of a reproducible test it is the number of cysts used that is important, and these should be distributed among three to four dishes. Experiments showed that increasing the volume of solution above 1 ml had little effect, and 1 ml was usually used with 15–30 cysts/dish.

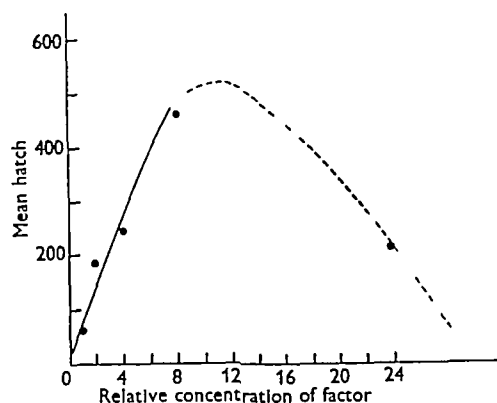


Fig 8 *Effect of hatching factor concentration on larval hatch*

A number of experiments was made on the effect of concentration of hatching factor on the hatching of cysts. In each case it was found that the number of larvae hatched was proportional to the concentration of factor up to a certain concentration,

above which the hatching decreased. The result of one such experiment is shown graphically in Fig 8. In the testing method which was finally adopted (see below) tests were always carried out on the first part of the curve, where concentrations are below those giving the maximum hatch, and it was assumed that the number of larvae which emerged was proportional to the concentration of the factor. It should, however, be noted that, on account of the high standard deviations usually obtained in proportion to the means, the hatch is a rather crude measure, even where a large number of dishes is used in each test (cf Table 7). It has, nevertheless, served as the basis of the test method which we have used throughout the work on the eelworm hatching factor described in this and succeeding papers. The testing procedure is given in detail below.

Testing of materials for hatching activity

The practice adopted has been to compare the activity of unknown materials with a standard preparation. For this purpose a large sample of crude solid from tomato root leachings was set aside at an early stage in the investigations. This material was obtained by evaporating the eluate from a charcoal adsorbate prepared, using 5 g charcoal/l of leachings and is the material described as Standard Solid throughout the present paper. It gave good hatches from viable cysts at dilutions of 1/20,000 to 1/40,000 and was allotted a Relative Activity (R.A.) of 1.

Tests were carried out as follows: a suitable number of cysts (usually 20), previously sorted and kept in water for 3–4 weeks at 23°, was counted into each dish (deep solid watch glasses are convenient) using a Pasteur pipette, the water was drained off with a fine pipette and replaced by 1 ml of the active solution. The dishes were now covered with squares of glass and incubated at 23–34° for 2–3 days, by which time 50–200 larvae had emerged in each dish. Counting was carried out directly under a low power microscope. Never less than three dishes were used and where possible four or five were preferred both for the unknown and the standard.

It occasionally happened that single dishes showed counts markedly different from the others in the same group, it was customary to reject any count which was greater than five times, or less than one fifth of, the other counts. Experience showed that the mean values were reasonably reliable, and when repeat estimations were carried out very similar results were usually obtained. After a test was completed, the cysts could be kept for a few days in water and used again, after several tests they tend to become mouldy and should then be discarded.

To estimate the activity of an unknown substance, it was dissolved in water at a suitable dilution and applied to one group of cysts, while to a control group a solution of Standard Solid at, say, 1/40,000 dilution

was added. After 2-3 days incubation, the emerged larvae were counted and from the result the R.A. of the unknown substance calculated.

In a typical experiment, an active concentrate was diluted to 1/2,000,000 and compared with Standard Solid at 1/40,000 with the following result

Standard 1/40,000 hatches (2 days, 15 cysts/dish)—144, 57, 112, 150, mean 116

Unknown 1/2,000,000 hatches (2 days, 15 cysts/dish)—165, 89, 163, 120, mean 134

From the result the unknown substance had R.A.

$$\frac{2,000,000 \times 134}{40,000 \times 115} = 58$$

It will be observed that a dilution was chosen (by trial and error) for the unknown substance which gave about the same hatch as the control. In these circumstances the importance of the ratio concentration/hatch was diminished, since two solutions giving the same hatch would be expected to contain the same concentration of factor.

In the past, workers have usually stated the maximum dilution at which materials show any activity. This is a very unsatisfactory method of expressing activity, partly because cysts vary in sensitivity and partly because it is difficult to decide the point at which hatching due to active material becomes significant, since even in water a few worms may hatch out. The test method we have described is easy to carry out and gives results quickly.

Experience in many trials has shown that it is fairly reliable, and occasions on which it has given misleading results have been very few. Its accuracy is not very high, but the great individual variation among eelworm cysts makes it difficult to achieve precision in assay. In the work described in this and succeeding papers we have employed mainly cysts from Chat Moss, Lancashire, and from Cambridgeshire (for which we are indebted to Messrs F. G. W. Jones and F. R. Petherbridge), we have also used successfully cysts isolated from soil in the Newcastle area obtained through the kindness of Dr C. Ellenby.

SUMMARY

1. A method is described for the preparation of crude solid concentrates of the potato eelworm hatching factor by leaching the roots of growing tomato plants with water, adsorbing the factor on charcoal and eluting with aqueous acetone.

2. The effect of various conditions on the hatching of eelworm cysts has been studied, and a reasonably reliable bioassay method for the hatching factor has been worked out.

We are deeply indebted to Prof. R. T. Leiper, F.R.S., for his advice and assistance, to Imperial Chemical Industries Ltd (Dyestuffs Division) and the Agricultural Research Council for their support of the investigations, and to Dr G. H. Orcutt of the Department of Applied Economics, Cambridge University, for statistical calculations.

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The Potato Eelworm Hatching Factor

2 PURIFICATION OF THE FACTOR BY ALKALOID SALT FRACTIONATION ANHYDROTETRONIC ACID AS AN ARTIFICIAL HATCHING AGENT

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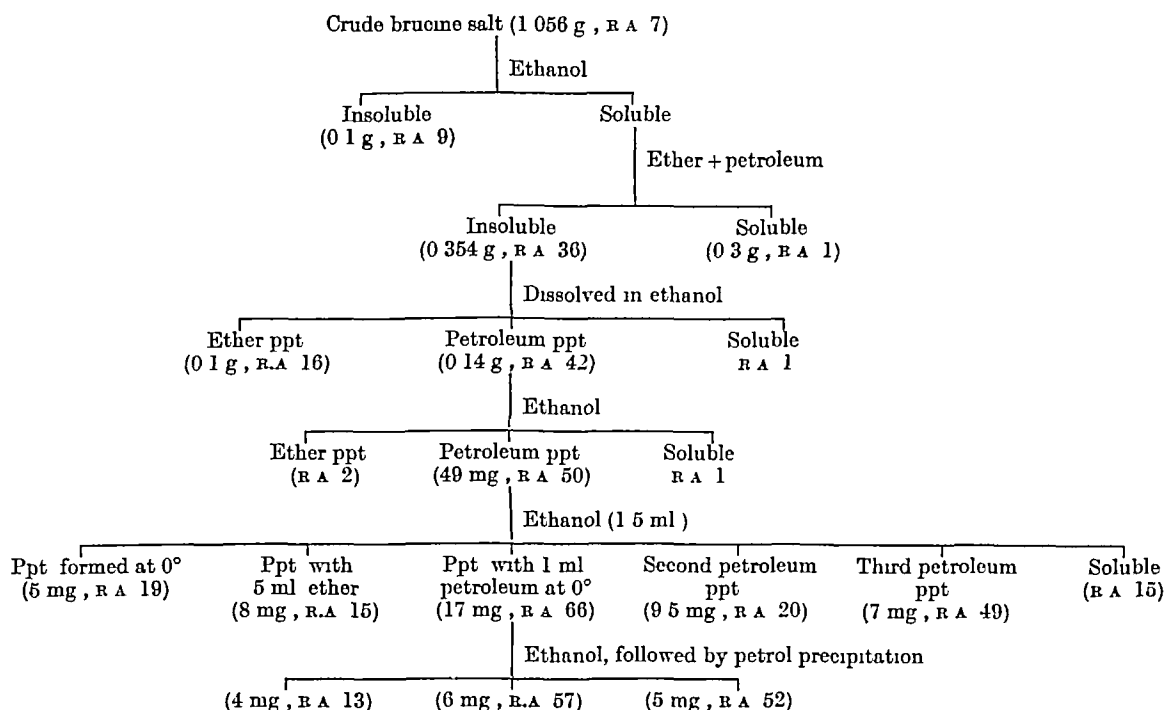
(Received 8 April 1949)

Methods for the preparation of crude solid concentrates of the potato eelworm hatching factor from leachings of tomato roots and for the bioassay of the factor being available as a result of work described in the preceding paper (Calam, Raistrick & Todd, 1949), experiments directed to the further concentration and eventual isolation of the active principle were undertaken. It was clear from preliminary experiments that, if only on account of a tendency to inactivation, attempted purification through metallic salts was unlikely to be successful, at least on the crude solid. Substantial purification could, however, be effected by extraction with organic solvents, thus from ether extracts of acidified aqueous solutions of crude solid a brownish glass was obtained having a Relative Activity (R.A.) about 25 and about one quarter the weight of the starting material. (Relative Activity is defined by Calam *et al.* 1949, p. 518.) This step, which results in little loss in activity, was accordingly included in all later concentration procedures. The product was strongly acidic and gave on titration an equivalent value of about 200, addition of excess alkali and back titration indicated the presence of lactonic material. It contained small amounts of nitrogen, but was free from phosphorus, halogens or sulphur.

As the product obtained by ether extraction could not be crystallized, and was almost certainly a complex mixture, it was treated with various alkaloids and the alkaloidal salts subjected to fractional precipitation. Brucine and quinine proved the most suitable bases, although neither was very satisfactory, as there was frequently complete loss of activity. It was noticed, too, that when the R.A. of a brucine salt fraction had reached about 50 it was difficult to raise it further without heavy loss. Although most of the earlier work was done on brucine salts, it was later found that quinine salts were equally useful. Examples of fractionations with both alkaloids are given below. The products were amorphous, but had fairly sharp melting points and gave reasonably consistent analytical values. The purest brucine salts showed R.A. 60–65. It was observed on a number of occasions that inactivation of the most highly active brucine salt fractions, on standing, on precipitation with solvents or on treatment with

a trace of alkali, yielded in nearly equivalent amount an inactive brucine salt which crystallized in rosettes of tiny needles and gave analytical values very close to those of the amorphous active salt. Such inactivation, with almost quantitative conversion to a crystalline salt, has not always been observed, perhaps because the amorphous active salts from different runs varied in their degree of heterogeneity. Treatment of the purified active brucine salts with acid, followed by continuous ether extraction, gave a highly acidic resin, active as hatching factor at dilutions of 1 in 10^7 to 1 in 10^8 , a very similar, although biologically inactive, resin was obtained by the same treatment applied to the inactive crystalline salt. The properties of these materials, so far as they were examined, are recorded in the experimental section. The evidently heterogeneous products were so intractable, and the amounts available for study at any one time were so small, that it would be unwise to draw any conclusions beyond that the hatching factor—for which we propose the name *eclepic acid*—is an acid probably containing a lactone group. It was decided to defer further investigation of the natural material until larger amounts could be made available.

Since the hatching factor is obtained by leaching the roots of growing potato and tomato plants, a number of substances which might occur in plant roots were tested for possible hatching activity. They included aneurin, riboflavin, nicotinamide, pantothenic acid, pyridoxin, liver and yeast concentrates of the other B vitamins, ascorbic acid, heteroauxin, traumatic acid and a urine concentrate which was believed to contain auxins *a* and *b*, none of them showed any activity. Contrary to the statements of Hurst (1937), who was probably misled by the biological test method he used, tyrosine, glutamic acid, lysine picrate and picric acid were found to have negligible activity. The high acidity and probable lactonic nature of *eclepic acid*, however, led us to test also a number of tetrone acid derivatives and other compounds, viz. carolic acid (Clutterbuck, Raistrick & Reuter, 1935), tetrone acid (β hydroxy $\Delta^{\alpha\beta}$ butenolide), α methyltetrone acid, α benzyltetrone acid, α carboxymethyltetrone acid, α acetyltetrone acid, α bromotetrone acid,

Table 1 *Fractionation of crude active brucine salts*

additional concealed acid group, possibly lactonic in nature. A molecular weight determination by the Barger method gave a value of about 300, but excessive dilution of the solutions which had to be used made the result unreliable (Found in material dried at 50°/0.1 mm over P_2O_5 , C, 55.7, H, 5.7%) The product gave no reaction for carbonyl groups, and catalytic hydrogenation gave a variable uptake of hydrogen corresponding to that required for 1 double bond in a molecular weight of 300–400. The free acid showed only very feeble optical activity in dilute aqueous solution ($[\alpha]_D$ about $+1^\circ$).

Synthesis of tetronic acid derivatives

Methyltetronate Excess ethereal diazomethane was added to tetronic acid and when evolution of N_2 ceased the solution was evaporated. Recrystallization of the residue from benzene/light petroleum and finally from a small amount of benzene gave the ester as colourless needles, m p 67° (Found C, 52.3, H, 5.3. $C_6H_8O_3$ requires C, 52.6, H, 5.3%)

Methyl α methyltetronate Methylation of α methyltetronic acid with diazomethane in the usual manner gave the methyl ester as a colourless liquid which distilled at $160\text{--}170^\circ/15$ mm (Found C, 56.3, H, 6.4. $C_6H_8O_3$ requires C, 56.2, H, 6.3%)

Methyl α benzyltetronate Methylation of α benzyltetronic acid with diazomethane gave the methyl ester as a colourless liquid, b p $155\text{--}160^\circ/5 \times 10^{-3}$ mm (Found C, 70.1, H, 6.0. $C_{12}H_{12}O_3$ requires C, 70.6, H, 5.9%)

Acetyl α benzyltetronate α Benzyltetronic acid was acetylated by heating on the steam bath with excess acetic anhydride for 2 hr. Acetic anhydride and acetic acid were removed under reduced pressure and the residue recrystallized from cold methanol. The acetyl derivative formed

colourless needles, m p $69\text{--}70^\circ$ (Found C, 67.1, H, 5.4. $C_{13}H_{12}O_4$ requires C, 67.2, H, 5.2%)

α Carboethoxy γ γ -dimethyltetronic acid Diethyl malonate (32 g) dissolved in dry ether (100 ml) was allowed to react with sodium wire (4.6 g) overnight. To the cooled mixture α bromoisobutyryl bromide (23 g) dissolved in dry ether (100 ml) was added, after 24 hr dilute H_2SO_4 (100 ml. of 3N) was added and the aqueous layer separated and evaporated under reduced pressure at room temperature. The sticky solid was purified by precipitation from benzene solution with light petroleum and finally recrystallized from a minimum of water. The acid formed colourless needles, m p $83\text{--}84^\circ$ (Found C, 53.6, H, 6.0. $C_8H_8O_5$ requires C, 54.0, H, 6.0%)

O Tetra-acetyl β glucosidyl α methyltetronate Dry powdered silver α methyltetronate (2.4 g) was added to a solution of α acetobromoglucose (4.1 g) in dry benzene (10 ml), and the mixture shaken for 10 hr. AgBr was removed and the filtrate set aside. The glucoside slowly separated as colourless needles and, after recrystallization from benzene/light petroleum, had m p $160\text{--}161^\circ$ (Found C, 51.6, H, 5.5. $C_{18}H_{22}O_{12}$ requires C, 51.3, H, 5.4%)

The hatching of eelworm cysts by anhydrotetronic acid

Anhydrotetronic acid is strongly acidic and differs from eclepic acid in that it is only active at pH values below 3. The results of a typical experiment in which the behaviour of anhydrotetronic acid was compared with the natural substance are summarized in Table 2.

Table 2 *Hatching of eelworms by anhydrotetrone acid at different pH values*

(The hatches are means of three dishes, each containing thirteen cysts incubated 3 days at 22°)

Anhydrotetrone acid (1/2000)		Standard solid (R.A. 1) from tomato root leachings (1/30,000)	
pH	Hatch (larvae)	pH	Hatch (larvae)
2.65	83	3.22	83
4.40	10	4.82	93
5.76	3	6.67	71
6.98	2	6.98	19
8.03	10	8.04	21

The structure (I) of anhydrotetrone acid gives no explanation for this behaviour. The absorption spectrum does not change on dissolution in alkali, and alkaline solutions become active once more on acidification. It would thus appear that the hatching mechanism can only be started by anhydrotetrone acid over a narrower pH range than by the natural substance. From the above experiment it can be seen that anhydrotetrone acid has an R.A. of about 0.07 compared with over 200 for eclepic acid.

When cysts are exposed to anhydrotetrone acid, the larvae emerge in the usual way (cf. Calam *et al.* 1949) being coiled and moving freely. This contrasts with the effect produced by certain other substances, such as bleaching powder solution or picric acid, which cause larvae to emerge in an angular, lifeless condition, and so can hardly be described as true hatching agents.

The results of an experiment in which eelworm cysts were mixed with sand and treated with anhydrotetrone acid for 35 days is summarized in Table 3.

Sand (100 ml.) washed with HCl and water was placed in glass jars and 100 cysts and 20 ml. water well mixed with it. The anhydrotetrone acid was dissolved in water and added after 1 week. The jars were kept at 15–20° in a room where they were not exposed to sunlight, and were occasionally watered to keep the contents moist. It was noticed that with higher concentrations of anhydrotetrone acid the upper layer of sand became tinged with purple. Soil could not be used because it was impossible with it to reduce the pH to 3 or less, and even with 600 mg. of anhydrotetrone acid per jar the sand mixture only just maintained this level. The jars were examined after 35 days.

It will be seen that where anhydrotetrone acid was used the cysts were no longer viable, so that the degree of hatching was perhaps less than it might otherwise have been. In another set of jars, which were kept for 92 days, even the untreated cysts were non-viable, suggesting that complicating factors may have been operative. Under normal conditions, cysts will continue to hatch for long periods without dying. Thus when 135 cysts containing 13,635 larvae were placed, for a few days, in hatching factor solution and water alternately, 6466 larvae emerged during the next 4 months and more were still slowly hatching. Dissection of a number of cysts at this stage showed that all had contributed some larvae, maximal and minimal numbers per cyst remaining being 223 and 36 at the start and 130 and 5 afterwards. It would appear that in the above experiment with sand some unknown factor was causing the death of the cysts.

Table 3 *Reduction in content of cysts by anhydrotetrone acid*

(Readings after 35 days, initial content, 101.6 larvae)

Anhydrotetrone acid		pH of extract	Acid recovered		Hatchability of recovered cysts	No. of cysts recovered	Larval content of recovered cysts	Mean reduction in larval content
(mg./jar)	(cwt./acre)		Titration (mg.)	Hatching test				
0	0	4.2	0	—	+	84	97.9	
0	0	4.3	0			93	105.8	
0	0	4.3	0			52	103.0	
0	0	4.4	0			79	92.0	
Mean		4.3	0			77	99.8	1.8
50	6.7	3.75	27			84	97.6	
50	6.7	3.95	26	—	—	70	104.0	
50	6.7	3.85	30			84	90.3	
Mean		3.85	28			79.3	97.3	4.3
200	27	3.45	77			82	97.9	
200	27	3.5	98	±	—	93	93.1	
200	27	3.4	115			92	95.7	
Mean		3.45	97			89	95.6	6.0
600	80	2.85	300			105	77.3	
600	80	2.85	365	++	—	95	92.8	
600	80	2.75	362			107	88.5	
Mean		2.8	342			102.3	86.2	15.4

From Table 3 it will be seen that the reduction in number of larvae per cyst after 35 days was 1.8, 4.3, 6.0 and 15.4 with 0, 50, 200 and 600 mg anhydrotetrone acid per jar. Statistical analysis shows that there is, in fact, a significant correlation between amount of anhydrotetrone acid added and the reduction in number of larvae. When a parallel series of jars were examined after 92 days a similar effect was observed.

SUMMARY

1 The potato eelworm hatching factor can be extracted with difficulty from acid aqueous solution by ether, and this process has been employed as a first stage in the purification of crude concentrates from tomato root leachings. The hatching factor is an acid, and for it the name eclepic acid is proposed.

2 The preparation of highly active brucine and quinine salts from eclepic acid concentrates is described, as also are some of the properties of the resinous free acid prepared from them. The most active preparations of eclepic acid, although still impure, show full hatching activity on eelworm cysts at a dilution of 1 in 10^7 to 1 in 10^8 .

3 No appreciable hatching activity was shown by a variety of substances likely to occur in plant leachings or by several amino acids and tetrone acids. Anhydrotetrone acid, however, was found to possess definite hatching properties, being fully active at a dilution of 1/2000. A preliminary pot experiment showed that it could bring about artificial hatching of eelworm cysts in sand.

Our thanks are due to the Imperial Chemical Industries Ltd. and to the Agricultural Research Council for the provision of grants and other assistance.

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The Potato Eelworm Hatching Factor

3 CONCENTRATION OF THE FACTOR BY CHROMATOGRAPHY OBSERVATIONS ON THE NATURE OF ECLEPIC ACID

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(Received 8 April 1949)

From the preliminary investigations described in Part 2 of this series (Calam, Todd & Waring, 1949) it was clear that unless considerably larger amounts of starting material could be made available, little progress towards the isolation and structural elucidation of the potato eelworm hatching factor (eclepic acid) was likely. Although the factor evidently had a comparatively low molecular weight, the small amount of it present in tomato root leachings, coupled with its great instability, made chemical investigation extremely difficult. Fortunately, larger supplies became available through the generous help afforded by the Agricultural Research Council, which arranged for the growing and leaching of tomato plants on a large scale by essentially the method described in Part 1 (Calam, Raistrick & Todd, 1949) to be carried out at the Experimental Station, Cheshunt. There a stock

of some 150,000 tomato plants was maintained under glass during the period May 1943 to September 1944. Production in 1944 was seriously affected by war damage at the station and ceased in the autumn of that year. The total amount of crude solid eluate obtained during the period was 218 g. with an average Relative Activity (R.A.) 9 (Calam, Raistrick & Todd, 1949), batches ranging from R.A. 4-13, under this arrangement then, we could extract about 12 g. crude solid per month, which, although small, was very much more than was available to us in our earlier work. The investigations described in this paper were all done on this material, referred to throughout as 'crude solid'. Assuming that R.A. about 200, the value shown by the most highly purified eclepic acid preparations obtained, represents something approaching the activity of pure eclepic acid, then on assay the total material we

received might contain 5–8 g. It has to be remembered, however, that the concentration procedure is always accompanied by heavy loss, and that the assumption regarding the activity of pure eclepic acid is wholly speculative, so that in fact the amount of factor obtainable in practice was much less than that which might be deduced from the bioassay figure for the crude solid.

From the early concentration studies of Calam, Todd and Waring (1949), it seemed clear that ether extraction from acid solution would be a valuable step in any isolation method, giving, as it does, a substantial reduction in weight without, as a rule, serious loss in activity. Accordingly, it was adopted as a standard first operation on crude solid in the present work. The alkaloid salt fractionation used in Part 2 of this series (Calam, Todd & Waring, 1949) was much less satisfactory, losses being heavy, and complete inactivation occurring at times for no obvious reason. It was, therefore, decided to examine adsorption methods more closely in the hope that one could be found which might effect a more far reaching concentration of active material before fractional precipitation of salts was used. Preliminary experiments (by Dr C. T. Calam) had shown that simple chromatography on alumina was of little value, as was indeed to be expected from the general properties of the active material. Adsorption of active material from aqueous solution at neutral and acid pH on kieselguhr, kaolin, fuller's earth and charcoal was then examined, of these adsorbents only charcoal was really effective, even when the ratio of adsorbent to material used was as high as 200/1. Attention was therefore turned to partition chromatography. At first the methods of Martin & Synge (1941) and Gordon, Martin & Synge (1943) were tried, using a silica gel column impregnated with methyl orange and applying to it the acid ether extract of the crude solid. Results were, however, unsatisfactory, as activity tended to spread over the column giving very indistinct banding. More success was achieved using a modification of this technique in which the chromatogram was a silica gel column loaded with strong potassium phosphate buffer at a suitable pH.

We are indebted to Dr A. A. Levi for allowing us to take advantage of this method, originally devised for work in the penicillin field, and as a result being unavailable through normal publication channels until later (Levi, 1948).

As a result of many trial experiments, a standard procedure of chromatography on a column buffered at pH 6 was adopted. The main active fractions from such chromatograms were resins showing a 3 to 4 fold increase in activity over the starting material. For further purification these products were treated with brucine, and the brucine salts shaken in aqueous solution with chloroform. The active salts passed

into the chloroform layer and could be obtained as amorphous white powders by final precipitation from ethanolic solution with light petroleum. These salts gave reasonably consistent analytical results, as did the resinous eclepic acid preparations made from them.

This revised concentration procedure was found to be in general more reliable than the method employed earlier, and the final products had R.A. 200–240 as free acid and R.A. 40–50 as brucine salts. Some of the brucine salts described in Part 2 (Calam, Todd & Waring, 1949) showed R.A. 60–65, but gave similar analytical results, since the tests were carried out at different periods and, bearing in mind the variability encountered in cyst hatching, we do not consider that this difference in R.A. is necessarily significant.

EXPERIMENTAL

Preparation of columns

In general, the method followed that described by Gordon *et al.* (1943). A weighed amount of the prepared silica (about 60 g/g of substance to be chromatographed) was dried overnight at 110° in an electric oven, and then stirred in a large basin with half its weight of strong phosphate buffer (30%) of the appropriate pH, which was added dropwise from a dropping pipette. When all the buffer had been added, the silica was mechanically stirred with wet CHCl_3 until the small lumps had been broken up and then poured in the usual manner into a glass column plugged with a little cotton wool. The column was washed with wet CHCl_3 and then with wet ether. When the entire column had been washed with ether, the material to be chromatographed was added in wet ether.

Partition chromatography of eclepic acid concentrates

The material submitted to chromatography was in all cases the product obtained by continuous ether extraction of an acidified aqueous solution of 'crude solid' according to the method described by Calam, Todd & Waring (1949).

The most suitable pH for chromatography by the method of Levi (1948) depends on the stability of the substance under examination and its partition coefficient between buffer and solvent and must be determined empirically. It was known from earlier work that eclepic acid was relatively stable below pH 7, and it was found by trial that 70–75% of the active material passed from ether into phosphate buffer of pH 5. This distribution appeared to be of the right order and, after trials on a series of columns at pH's in this region, pH 6 was finally selected as the most suitable. On a column of pH 6 most of the material was firmly held, only a pale yellow inactive oil passing through with ether: this inactive oil had a terpene-like odour and is referred to as the 'terpene fraction'. The chromatogram could be developed with acetone ether mixtures. Experience showed that there was some spread of activity in the various fractions, but the majority of the active material was located in the 50% acetone ether runnings. When all the active material had been eluted, the silica was yellow in colour and this colour—probably due to an anthoxanthin colouring matter—could

only be removed with water. Many such chromatograms were studied, and finally the following method was adopted in which only three fractions were taken. The material to be purified was put on the column in wet ether and the inactive terpene fraction (usually 30–35%) collected in the ether runnings and washings. The column was now pushed out of the tube and cut in half. The upper half, which contained all the activity, was eluted by stirring with acetone ether (1:1) giving an active middle fraction (25–30%). The third, inactive, fraction containing yellow colouring matter could subsequently be eluted from the column with water. Typical results are given in Table 1. On some occasions there was found associated with the active fraction on the column a neutral biologically inactive crystalline compound, m.p. 136°. This substance appeared to have a formula $C_{12}H_{14}O_4$ on the basis of analysis and molecular weight determination, but its structure has not been determined.

Table 1 *Middle fractions from buffered chromatograms at pH 6*

Crude ether extract		Middle fraction		Wt recovery (%)
Wt (mg)	R.A.	Wt (mg)	R.A.	
208	25	82	70	40
327	36	109	126	30
412	30	153	98	37
1193	—	422	68	37
2150	22	650	80	30

Purification of middle fractions from chromatograms

Attempts were now made to purify the eclepic acid present in the middle fractions from chromatograms of the type discussed above by brucine salt fractionation. After numerous trials it was found that virtually colourless active salts could be obtained by extracting the mixed brucine salts in aqueous solution with $CHCl_3$ in which the active salts are readily soluble and then re-extracting from the $CHCl_3$ layer with suitable quantities of water. Active salts obtained in this way were then further purified by precipitation from ethanol with ether light petroleum mixtures. In this way amorphous brucine salts of R.A. 40–50 were obtained which gave consistent analyses. In the course of this work small amounts of three different inactive crystalline brucine salts were obtained, the first had m.p. 162–168° and appeared to be brucine formate, the second, m.p. 187–190°, probably brucine β -hydroxyglutamate and a third, m.p. 159–162°, which gave analytical values very similar to those given by the active salts.

From the purified active brucine salts, free eclepic acid was obtained as a yellowish resin by acidification and extraction with peroxide-free ether. This hygroscopic resinous product gave, like the parent brucine salts, fairly consistent analyses and usually had R.A. 200–240. During the preparation of the free acid, complete inactivation occasionally occurred, this unaccountable tendency to inactivation has been a major source of difficulty at all stages of work with the potato eelworm factor. The resinous acid defied all efforts at crystallization and could not be distilled without decomposition and loss of biological activity. A very large number of unsuccessful attempts was also made to obtain a crystalline salt or other derivative so as to get some evidence as to its homogeneity, e.g. Na, Ba, Ca,

Ag, Pt, benzylamine, acridine, benzylthiuronium salts, *p*-phenylphenacyl and other esters, amides, anilides, toluenes, hydrazides. The acid gave a transient reddish brown colour with $FeCl_3$ —a reaction which had apparently been missed in earlier work—but otherwise showed no characteristic colour reactions.

In Table 2 analytical figures are given for a series of active brucine salts, R.A. 40–50, and for the resinous eclepic acid preparations obtained from the salts. Agreement between individual analyses is surprisingly good considering the nature of the materials.

Table 2 *Analyses of amorphous active brucine salts and of the resinous eclepic acid prepared from them*

Sample no	Brucine salt (dried over P_2O_5 at 100°/0.1 mm)			Free acid (dried over P_2O_5 at 60°/0.1 mm)		
	C (%)	H (%)	N (%)	C (%)	H (%)	N (%)
1	63.3	6.7	5.7	57.0	6.2	—
2	63.9	6.6	6.2	55.6	6.3	1.6
3	63.2	6.3	6.2	55.9	5.6	2.2
4	62.7	6.9	6.6	57.0	6.4	1.5
5	63.6	6.5	5.8	55.9	5.9	2.5
6	63.4	6.5	6.2	—	—	—
Mean	63.4	6.6	6.1	56.4	6.1	2.0

The low but ever present amount of N in eclepic acid preparations was a continued source of confusion. Two possibilities had to be considered. The eclepic acid preparations, in view of their more or less consistent analyses and activity, might be nearly pure, in which case the mol. wt. would require to be of the order 700, or, alternatively, the N might be present in some contaminating material. On the whole, the second possibility seemed the more likely. Eclepic acid preparations showed no basic properties, were not inactivated by treatment with HNO_3 and yielded their N quantitatively in the form of NH_3 (identified as ammonium chloroplatinate) on hydrolysis at 100° with HCl. To throw further light on this point we decided to investigate the use of a second silica phosphate buffer chromatogram at more acid pH in place of the brucine salt fractionation. It was found that the active material present in the middle fraction from a pH 6 chromatogram could be made to travel down a chromatogram buffered at pH 4–4.5 if applied in ether containing 1% *n*-butanol and developed with ether containing increasing amounts of *n*-butanol. The most active fractions (R.A. about 200) from such chromatograms were those eluted with ether containing 4% *n*-butanol and they had N contents of 1% or even less. It is thus very tentatively concluded that eclepic acid is N-free, it being assumed on available evidence that the purest preparations obtained, although certainly not homogeneous, consist largely of the factor. The only other possibility is that the active material is a minor constituent of the preparations obtained, this seems rather unlikely, particularly since these preparations are fully active at dilutions as high as 1 in 10^7 .

DISCUSSION

In the absence of any real evidence for the homogeneity of the most highly purified samples of eclepic acid so far prepared, the interpretation of analytical or degradative data obtained using them is so

difficult that any conclusions can only be accepted with the greatest reserve. It is evident, from the analytical figures given for the free acid and its brucine salt in Table 2, that the material prepared by chromatography and salt fractionation was not homogeneous, but working on the assumption that it consisted largely of eclepic acid with minor amounts of impurity, we have carried out analytical and degradative experiments in the hope of finding some clue to the structure of the hatching factor. This assumption rests, of course, on the very high biological activity of the products and the fact that the carbon and hydrogen contents of samples prepared at different times from different batches of starting material were of the same order.

In general, titration of such materials indicated an equivalent of 250–290 and addition of excess alkali and back titration showed the presence of a lactone group (about 80 % of theory calculated on an equivalent of 250). From the ease of opening, the group concerned seems likely to be a γ lactonic group and the hydroxyl involved in its formation is not tertiary, the greater resistance of lactone rings involving tertiary hydroxyls was checked by parallel examination of a series of synthetic compounds of this type. Unfortunately, the solubility of eclepic acid prevented determination of molecular weight by the common methods. A rather unsatisfactory attempt on one sample using Barger's method gave a value of about 300. Catalytic hydrogenation using both platinum and palladium catalysts led to hydrogen uptake corresponding to one double bond in a molecular weight of 300–400 according to the sample of material used. The optical activity of the purified materials was similar to that determined for earlier samples in Part 2, but all purified concentrates gave a reddish brown colour with ethanolic ferric chloride suggesting the presence of an enolic (or phenolic) grouping. Quantitative acetylation gave rather variable results indicating one to two hydroxyl groups in a molecular weight of 300–400. Zerewitinoff determinations indicated two to three active hydrogen atoms in the same molecular weight range. The absorption spectrum of eclepic acid concentrates was not very characteristic, in ethanolic solution the absorption maximum which could not be accurately determined occurred below 2200 Å ($E_{1\text{cm}}^{1\%} > 30$), and there was a marked inflexion at 2850 Å (determination by Dr A. E. Gillam, Manchester University). These findings would not be inconsistent with the presence of a double bond in conjugation with a carboxyl and possibly an aromatic ring elsewhere in the molecule.

A variety of degradative experiments was carried out. Acid hydrolysis at various temperatures yielded no identifiable products other than traces of acetone, and alkaline hydrolysis was equally unsuccessful. The only identifiable product on oxidation with

alkaline potassium permanganate or chromic acid was acetic acid, whilst selenium dehydrogenation gave only intractable resins. Treatment with diazo methane yielded products containing nitrogen, presumably by addition of the reagent to some unsaturated centre, and the methyl ester prepared from the silver salt with methyl iodide could not be satisfactorily purified. Methylation with dimethyl sulphate in alkaline solution at 70° yielded more promising results, the product could not be crystallized, but it gave analytical figures corresponding to a formula $C_{19}H_{24}O_8$. (Found in two different samples C, 59.7, 59.1, H, 6.8, 6.4, MeO, 8.3. $C_{19}H_{24}O_8$ requires C, 59.7, H, 6.8, 1 MeO, 8.1 %). The methylated acid shows no ferric chloride reaction and is free from nitrogen. It is of some interest to note that if the formula $C_{19}H_{24}O_8$ can be applied to this material it would be the methylation product of an acid $C_{18}H_{24}O_8$ whose carbon and hydrogen contents (C, 58.7, H, 6.5 %) are not widely different from those of our best eclepic acid preparations. Such a formula would be in reasonable accord with such evidence as is available regarding the molecular weight of the hatching factor and could accommodate the necessary carboxyl group, lactone group, double bond and two hydroxyls, of which one has acidic properties.

Some further degradative experiments have been carried out with the methylated acid described above, but discussion of them is deferred since, until an eclepic acid preparation is obtained which is known to be homogeneous, arguments based on degradative evidence may be very misleading and little weight can be attached to any formula for eclepic acid based on them. It is hoped that fresh supplies of raw material will enable us to prepare a pure specimen of eclepic acid so that progress can be made in its structural elucidation.

SUMMARY

1 The application of two phase chromatography on silica gel loaded with strong phosphate buffer to the concentration of the potato eelworm hatching factor is described. Chromatography at pH 6 followed by brucine salt fractionation, or two stage chromatography at pH 5.5–6 and 4–4.5, effect similar degrees of concentration, the resinous products being fully active in hatching eelworm cysts at dilutions of 1 in 10^7 .

2 The composition and chemical properties of the most highly purified hatching factor concentrates are discussed.

Grants in aid of this work from the Agricultural Research Council and Imperial Chemical Industries Ltd. (Dyestuffs Division) are gratefully acknowledged. We are also greatly indebted to Dr Owen of the Cheshunt Experimental Station for his invaluable work in growing and leaching the tomato plants necessary for the investigation.

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The Potato Eelworm Hatching Factor

4 *SOLANUM NIGRUM* AS A SOURCE OF THE POTATO EELWORM HATCHING FACTOR

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It has been known for some time that the root excretion of several species of *Solanum* stimulates the hatching of larvae from the cysts of the potato eelworm, *Heterodera rostochiensis* (Franklin, 1940). The isolation of the active principle in root excretion and the investigation of its chemical nature was first attempted, using the leachings from potato plants (Hurst, 1935, 1937), but the method of production was cumbersome and a more convenient source of material was found in tomato plants. The tomato plants were more easily grown indoors than potatoes, and gave a very active excretion. As work on the chemical nature of the active principle developed (Calam, Raistrick & Todd, 1949; Marrian, Russell, Todd & Waring, 1949), greater demands were made upon production of starting material for the investigation, and necessitated the growing of many thousands of tomato plants during a season. It became clear that if the scale of the work continued to expand a new source of root excretion, which could be easily and cheaply grown out of doors on a large scale, would be welcomed to replace the growing of tomato plants under glass. With this end in view, a small scale outdoor experiment with *Solanum nigrum* was carried out during 1943 at the suggestion of Sir E J Salisbury, F R S, who also very kindly supplied the seeds, and the yield, quality and nature of the root excretion were examined.

METHODS

The growing and leaching of Solanum nigrum plants

The plants were grown from seed in two trays A and B (each 4 × 3 ft) having an inclined base of asbestos board which facilitated rapid drainage of leachings into a gutter of the same material leading to a large collecting jar. A layer of

broken brick ($\frac{1}{2}$ in deep) was laid at the bottom of each tray, and thus was covered with a mixture of garden soil and sand (tray A contained 2 parts soil to 1 part sand to a depth of 4–5 in. and tray B 4 parts soil to 1 of sand). The seeds were sown in April 1943, in rows 5 in. apart, and the seedlings thinned out 6 weeks later, leaving about 120–130 plants in each tray. Towards the end of June, when the plants were 4 in. high and growing vigorously, they were well watered and a sample of leachings tested for activity by the method already described (Calam *et al.* 1949). The leachings proved to be highly active, hatching 683 larvae from 60 cysts during 3 days. It was therefore decided to begin regular leaching of the plants at once.

The plants were leached early in the morning on alternate days, and the leachings treated at once with activated charcoal (British Drug Houses Ltd., 'for decolorizing purposes', 1 g/l), shaken and left to settle. On the following day the bulk of the liquid was decanted from the charcoal, which was then sucked fairly dry on a Buchner funnel. The moist charcoal was allowed to accumulate until the end of each week and was then eluted with aqueous acetone. Watering was controlled so as to give about 3–4 l of leachings from each tray every alternate day. In actual practice, however, this volume was rather variable as the plants were unprotected from the weather, and during rainy periods the trays were liable to be almost completely depleted of their content of hatching factor. The leachings were a very pale yellow and had pH 7.

RESULTS

Elution of charcoal

The accumulated moist charcoal on which the active factor was adsorbed was eluted each week with aqueous acetone (70 parts acetone to 30 parts water). About 40 g of charcoal were stirred four times with 100 ml aqueous acetone and the yellow filtrate concentrated below 30° under reduced pressure, and finally evaporated to dryness in a desiccator. The

crude solid eluate was a brown resinous powder and had a high activity when tested against an arbitrarily chosen 'standard solid' eluate from tomato plants (cf Calam *et al* 1949). As will be seen from Table 1 the Relative Activity (R.A.) of the crude eluate from tray A was very high during the early period of growth (R.A. 20-30) and gradually decreased after the plants had reached maturity. The weight yield of crude eluate per litre of leachings also showed a gradual decrease throughout the experiment, except in the last recorded week which showed a large increase in weight yield. As this increase occurred in the case of both trays at the same date, it was probably due to the exceptionally heavy rain fall during the previous week. The wet weather during September brought the experiment to a premature close, although it is doubtful whether much more could have been expected from the plants even if the weather had been more favourable.

The yield of crude solid from tray A was better in weight and activity than that from tray B (Tables 1 and 2). This may have been due to a variety of

Concentration of hatching factor

The purification of the crude solid eluate was carried out by applying the procedure which had been developed for the concentration of root excretion of tomato plants by Marrian *et al* (1949).

The crude solid (2.3 g) was dissolved in water, acidified with dilute H_2SO_4 and extracted continuously with peroxide free ether for 12 hr. The moist ether extract (150 ml.) was put directly on to a column prepared from 20 g silica gel moistened with 8 ml 30% phosphate buffer, pH 6.0. After washing with wet ether (150 ml) the column was drained and eluted by stirring with aqueous acetone (1 l). Evaporation of the ether runnings and washings gave 90 mg of sweet smelling oil, probably terpene material, while the acetone eluate yielded 75 mg of clear yellow resin, R.A. 150.

Brucine salt fractionation. The active concentrate from the chromatogram was dissolved in water (10 ml.) and shaken with brucine (250 mg). The undissolved brucine (100 mg) was filtered off, and the filtrate extracted by shaking several times with $CHCl_3$. Evaporation of the $CHCl_3$ extract gave 220 mg brucine salts which were dissolved in ethanol (2.5 ml) and a little insoluble matter spun off. Addition of ether

Table 1 *Details of hatching factor production from Solanum nigrum (tray A)*

Week ending	Volume of leachings (l.)	Wt moist charcoal (g.)	Wt crude eluate (mg.)	R.A.	Wt crude eluate (mg./l.)
8 July 1943	11.5	42	205	24	18
17 July 1943	12	28	130	28	11
23 July 1943	13.5	38	160	32	12
31 July 1943	15.5	41	133	10	9
6 Aug 1943	18	42	158	20	9
14 Aug 1943	18	50	161	7	9
25 Aug 1943	27.5	75	173	7	6
(Heavy rain all week ending 1 Sept 1943)					
13 Sept 1943	19	50	238	11	13
Total			1368		

Table 2 *Details of hatching factor production from Solanum nigrum (tray B)*

Week ending	Volume of leachings (l.)	Wt moist charcoal (g.)	Wt crude eluate (mg.)	R.A.	Wt crude eluate (mg./l.)
24 July 1943	9	26	123	—	14
30 July 1943	17	57	183	18	11
6 Aug 1943	11	26	103	10	9
14 Aug 1943	14.5	40	137	3	9
25 Aug 1943	27.5	81	156	3	6
(Heavy rain all week ending 1 Sept 1943)					
13 Sept 1943	17	55	241	5	14
Total			943		

reasons. Tray A was planted some 3 weeks earlier than tray B and contained rather more plants. The plants in tray A therefore had a more favourable growing period as the later part of the summer was rather wet and cold. Another difference between the two trays was that B contained less sand than A, and consequently drained more slowly. The total yield of crude eluate obtained from both trays (251 plants) during the experiment was 2.3 g.

caused no precipitate, so a large excess of light petroleum was added (20 ml) and the precipitated brucine salt (63 mg) was collected and analysed, R.A. 35. (Found in material dried *in vacuo* over P_2O_5 at 100° C, 65.1, H, 6.5, N, 5.8%. Average value for corresponding product from tomatoes C, 63.4, H, 6.6, N, 6.1%.)

Preparation of the inactivated free acid. The purified brucine salt (60 mg) was dissolved in water, acidified with dilute H_2SO_4 and extracted continuously with ether for 20 hr. Evaporation of the ether extract left 16 mg of free acid which

was inactive (Found in material dried *in vacuo* over P_2O_5 at $50^\circ C$, 60 0, H, 6 1, N, 2 0% Average value for corresponding product from tomatoes C, 56 4, H, 6 1, N, 1 8%)

CONCLUSIONS

The growing of *Solanum nigrum* out of doors with the minimum amount of attention yielded a root excretion which was highly active in hatching larvae of the potato eelworm from their cysts. Unfortunately, the rapid growth and short growing period of the plants did not permit more than about 9 weeks' leachings to be collected. The active principle from *S. nigrum* root excretion could be concentrated by the same procedure as was used for tomato root excretion, but the limited number of analyses on the brucine salt and free acid did not justify the deduction that the active principle from *S. nigrum* was identical with that obtained from tomatoes. Nevertheless, making allowance for the

non crystalline character of the products, the great similarity in general properties would make it appear probable that the *S. nigrum* factor is either identical with that obtained from tomatoes or some very closely related substance.

SUMMARY

1 *Solanum nigrum* grown out of doors yielded a root excretion which was highly active in hatching potato eelworm cysts.

2 Concentration of the active material in this excretion gave a product which was closely similar to the corresponding product from tomato root excretion in its properties. The active principle in the two cases may well be identical.

We are grateful to Imperial Chemical Industries Ltd., and to the Agricultural Research Council, for grants in aid of this work. We wish to thank Sir E. J. Salisbury, F.R.S., for his interest and advice.

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The Potato Eelworm Hatching Factor

5 ATTEMPTS TO PREPARE ARTIFICIAL HATCHING AGENTS PART I. SOME FURAN DERIVATIVES

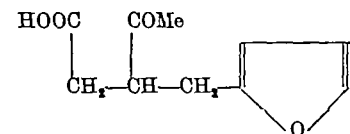
By P. B. RUSSELL, A. R. TODD AND W. S. WARING
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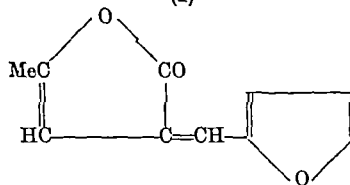
From the time when it became clear that highly active concentrates of the potato eelworm hatching factor prepared from tomato root excretion possesses both acidic and lactonic properties (Calam, Todd & Waring, 1949), synthetic experiments have been carried out alongside work on the natural factor in the hope that simpler substances might be prepared which would show hatching activity and which might be of practical interest in controlling eelworm infestation in potato crops. The examination of a number of tetrone acid derivatives has been described in a previous paper (Calam, Todd & Waring, 1949), the present communication deals with experiments on the synthesis of a number of lactones containing furan nuclei, such compounds being of interest, since it seemed probable that the natural hatching factor contained heterocyclic oxygen.

In commencing studies in the furan series an attempt was made to oxidize furitaconic acid (furfurylidenesuccinic acid) to β -hydroxy β -carboxy- γ -2-furylbutanolide by means of potassium permanganate or monoperphthalic acid without result, this was unexpected in view of the ease with which itaconic acid yields hydroxyparaconic acid on similar treatment. Condensation of furfural with laevulinic acid in presence of sodium acetate gives a 10% yield of β -furfurylidene laevulinic acid (Kehrer & Kleberg, 1893). Replacement of sodium acetate by piperidine failed to give any condensation and sodium hydroxide gave sticky products, with potassium acetate, however, yields of about 30% were obtained. No condensation occurred between furfural and ethyl laevulinate in presence of piperidine, but with sodium hydroxide β -furfurylidene-

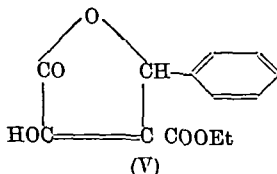
laevulinic acid was produced in moderate yield. Reduction of β furfurylidene laevulinic acid with sodium amalgam gave, according to conditions, β furfuryl laevulinic acid (I) or β furfuryl- γ methyl butanolide (II). Attempts to prepare analogous compounds by condensing ethyl 5 methyl 2 formyl-furan 4 carboxylate with laevulinic acid failed.



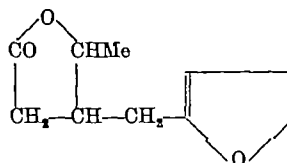
(I)



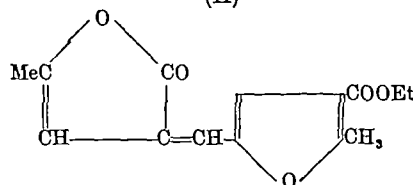
(III)



(V)



(II)



(IV)

Condensation of furfural with Δ^{β} γ angelicalactone gave α furfurylidene- γ methyl Δ^{β} γ -butanolide (III), hydrolysed to α furfurylidene laevulinic acid and reduced by sodium amalgam to α furfuryl- γ methyl butanolide. On refluxing α furfurylidene laevulinic acid with acetic anhydride it was reconverted to (III) in good yield. In similar fashion ethyl 5 methyl 2 formylfuran 4 carboxylate and Δ^{β} γ angelicalactone gave α (5 methyl-4 carboxyfurfurylidene) γ methyl Δ^{β} γ butanolide (IV). This lactone was readily hydrolysed to α (5 methyl-4 carboxyfurfurylidene) laevulinic acid, which gave α (5 methyl-4-carboxyfurfurylidene)- γ methyl Δ^{β} γ butanolide on refluxing with acetic anhydride. On reduction with sodium amalgam the original lactone (IV) gave as the only pure product α (5 methyl-4 carboxyfurfuryl) laevulinic acid. Attempts to condense furfural with ethyl sodio-oxaloacetate gave only resinous products, although benzaldehyde condensed readily enough, yielding the crystalline α hydroxy β -carbethoxy- γ phenyl Δ^{α} β butanolide (V, cf Gault & Durand, 1943).

All the above lactones and the corresponding laevulinic acid derivatives were tested for hatching activity on potato eelworm cysts by the technique described elsewhere (Calam, Raistrick & Todd, 1949), but in no case was any activity detected. Further synthetic work in this series was therefore abandoned, especially as the discovery that anhydrotetrone acid

shows hatching activity had suggested other possible lines of development.

EXPERIMENTAL

β Furfuryl laevulinic acid β Furfurylidene laevulinic acid (2 g) was dissolved in aqueous NaOH (10 ml of 3N), diluted

with water (200 ml) and Na amalgam (50 g of 4%, 2 mol) added with shaking. After a time the solution became colourless and it was then acidified, extracted with ether and the extract dried and evaporated. The crystalline product was purified by sublimation at 70°/10⁻⁴ mm (bath temp). Colourless needles, m.p. 98-99° (Found C, 60.8, H, 6.2 C₁₀H₁₂O₄ requires C, 61.2, H, 6.1%).

β Furfuryl γ methylbutanolide β Furfurylidene laevulinic acid was dissolved in aqueous NaOH (50 ml of 3N) and excess Na amalgam (150 g of 4%, 6 mol) added. The mixture, which became colourless in 1 hr, was shaken overnight and then acidified and extracted with ether. The ethereal extract was thoroughly washed with NaHCO₃ and water, dried and evaporated. The residual lactone (1.8 g), a colourless oil, was purified by distillation and had b.p. 70-75°/10⁻¹ mm (bath temp), at atmospheric pressure it had b.p. 230° (Found C, 60.2, H, 6.7, equiv (lactone), 183 C₁₀H₁₂O₃ requires C, 60.7, H, 6.7% equiv (lactone), 180). On refluxing the lactone with hydrazine hydrate in ethanol it yielded the hydrazide of γ hydroxy β furfurylvaleric acid crystallizing from ethyl acetate as colourless plates, m.p. 153-154° (Found N, 13.5 C₁₀H₁₂O₃N₂ requires N, 13.2%).

α Furfurylidene- γ methyl Δ^{β} γ butanolide A mixture of furfural (1 g) Δ^{β} γ angelicalactone (1 g) and piperidine (5 drops) was warmed on the steam bath for 1 hr and then allowed to cool. The yellow solid was washed with a little ether and recrystallized from methanol. The yellow needles, m.p. 78-79°, had a slight brownish tinge which could be removed by sublimation in vacuo (Found C, 68.0, H, 4.7 C₁₀H₈O₃ requires C, 68.1, H, 4.5%).

α *Furfurylidene laevulinic acid* The above lactone (1 g) was refluxed with excess methanolic KOH (3%) during 3 hr. Most of the methanol was then distilled off and the residue diluted with water and acidified. The yellow oil which separated was dissolved in a minimum of hot aqueous ethanol. On cooling α *furfurylidene laevulinic acid* separated as colourless needles, m p 125–126° (Found C, 62.3, H, 5.4, $C_{10}H_{10}O_4$ requires C, 61.8, H, 5.1%) On refluxing with acetic anhydride (3 ml) for 2 hr the acid (0.1 g) was reconverted to the above butenolide (0.00 g), m p 78–79°.

α *Furfuryl γ methylbutanolide* The above unsaturated lactone (2 g) was dissolved in aqueous ethanol (50 ml of 90%) and Na amalgam (150 g of 4%, 6 mol) added. The mixture was shaken until colourless (about 1 hr) then acidified and ethanol removed by evaporation under reduced pressure. The residual solution was extracted with ether and the extract washed with $NaHCO_3$, dried and evaporated. The pale yellow residual oil distilled at 85°/10⁻¹ mm (bath temp) (Found C, 66.4, H, 6.5, equiv (lactone), 180, $C_{10}H_{12}O_3$ requires C, 66.7, H, 6.7%, equiv (lactone), 180).

Refluxed with hydrazine hydrate in ethanol the product gave the *hydrazide* of γ hydroxy α *furfurylvaleric acid* as colourless needles (ethyl acetate), m p 138–139° (Found C, 56.6, H, 7.5, N, 13.6, $C_{10}H_{12}O_5N_2$ requires C, 56.6, H, 7.5, N, 13.2%).

Ethyl 5 methyl 2 formylfuran-4-carboxylate Prepared by oxidation of ethyl 5 methyl 2 (*d* arabinotetrahydroxybutyl) furan-4-carboxylate (West, 1925, Gonzalez, 1934), the aldehyde had m p 56° and gave a 2,4-dinitrophenylhydrazone, m p 220–221°. Unknown to us at the time this work was carried out, it had also been prepared by Müller & Varga (1939), and was subsequently described by Jones (1945).

α (5 *Methyl-4-carbethoxyfurfurylidene*)- γ methyl Δ^{β} γ *butenolide* Prepared in similar fashion to the analogous α *furfurylidene lactone* by condensing Δ^{β} γ angelicalactone with ethyl 5 methyl 2 formylfuran-4-carboxylate. Yellow needles, m p 143–144° from methanol (Found C, 63.8, H, 5.4, $C_{14}H_{14}O_6$ requires C, 64.1, H, 5.3%) Reduction in aqueous ethanol with Na amalgam in the usual manner gave α (5 *Methyl-4-carboxyfurfuryl*) *laevulinic acid* crystallizing from water in colourless needles, m p 126–129° (Found C, 56.4, H, 5.6, $C_{12}H_{14}O_6$ requires C, 56.7, H, 5.5%) Treatment of the gummy neutral fraction (which gave a positive Legal reaction) from the reduction mixture with hydrazine

gave a colourless product, m p 218–219°, which from analysis appeared to be the *pyridazine* corresponding to α (5 methyl-4-carboxyfurfuryl) *laevulinic acid* (Found C, 57.7, H, 5.5, N, 11.0, $C_{11}H_{14}O_4N_2$ requires C, 57.6, H, 5.6, N, 11.2%).

α (5 *Methyl 4-carboxyfurfurylidene*) *laevulinic acid* The foregoing ester lactone (0.5 g) was refluxed with methanolic KOH (3%) for 3 hr, the bulk of the methanol removed under reduced pressure and the residue diluted with water and acidified. The acid separated from aqueous ethanol as a yellow microcrystalline powder, m p 225–226° (Found C, 57.4, H, 5.0, equiv, 125, $C_{11}H_{12}O_6$ requires C, 57.1, H, 4.8%, equiv, 126).

α (5 *Methyl 4-carboxyfurfurylidene*) γ methyl Δ^{β} γ *butenolide* The above acid (0.5 g) was refluxed with acetic anhydride (3 ml) during 4 hr. The mixture was poured into ice water and the brown solid product crystallized several times from methanol. Small yellow needles, m p 210–220° (Found C, 61.5, H, 4.1, $C_{11}H_{10}O_6$ requires C, 61.5, H, 4.3%).

α *Hydroxy β carbethoxy γ phenyl Δ^{α} β -butenolide* Ethyl sodio oxaloacetate (43 g, 1 mol) was ground to a paste with ethanol (7 ml) and water (20 ml), the mixture cooled in ice and a solution of freshly distilled benzaldehyde (21.2 g, 1 mol) in ethanol (5 ml) added. After 30 min the reaction mixture was removed from the ice bath and left overnight at room temperature. Acidification with HCl gave an oil which was taken up in ether, the extract freed from any unchanged benzaldehyde with $NaHSO_3$, washed and evaporated. The solid residue crystallized from aqueous methanol in colourless needles, m p 104–105° (Found C, 62.7, H, 4.8, $C_{13}H_{14}O_6$ requires C, 62.9, H, 4.8%) The compound gave a positive reaction with $FeCl_3$, but had no ketonic properties.

SUMMARY

A number of substituted lactones and laevulinic acid derivatives containing furan nuclei have been synthesized. None of the compounds showed any activity when tested as hatching agents for the potato eelworm.

The authors' thanks are due to the Agricultural Research Council and Imperial Chemical Industries Ltd for their support of this investigation.

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The Potato Eelworm Hatching Factor

6 ATTEMPTS TO PREPARE ARTIFICIAL HATCHING AGENTS PART II SOME ACTIVE ARYLIDENE Δ^8 γ -BUTENOLIDES AND RELATED COMPOUNDS

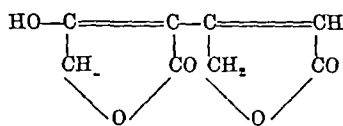
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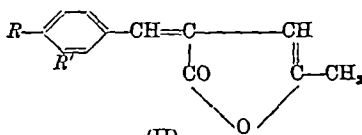
In a previous paper (Russell, Todd & Waring, 1949) the synthesis of a number of furan derivatives for examination as possible hatching agents for cysts of the potato eelworm (*Heterodera rostochiensis* Wollen weber) was reported. None of these materials showed any activity, indeed, out of a large number of substances, both synthetic and natural, belonging to a wide variety of chemical types, only anhydrotetronic acid (I) showed any appreciable activity (Calam, Todd & Waring, 1949). In the course of establishing the structure of anhydrotetronic acid (Marrian, Russell, Todd & Waring, 1947) it was shown that γ substituted tetronic acids do not yield substituted anhydrotetronic acids, so that the more obvious homologues and analogues of (I) are not readily obtainable. In these circumstances the search for synthetic hatching agents was directed to the preparation of rather different types of compounds, the molecules of which, however, contained conjugated systems analogous to that present in anhydrotetronic acid, e.g. compounds based on α arylidene Δ^8 γ butenolides. The present memoir describes some of this work which has led to the discovery of several biologically active compounds

being replaced by a methylenedioxy group). As previously reported, the same general method has been utilized for the preparation of unsaturated lactones containing furan nuclei (Russell *et al.* 1949).

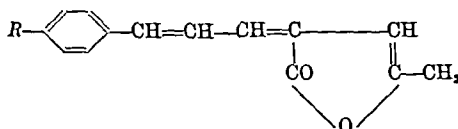
Condensation of *p* hydroxybenzaldehyde with Δ^8 γ angelicalactone in the absence of solvent but in presence of a little piperidine, triethylamine or methylmorpholine, gave α (*p* hydroxybenzylidene)- γ methyl Δ^8 γ butenolide (II, $R=OH$, $R'=H$). With *m* hydroxybenzaldehyde the reaction had to be carried out in ethanol as solvent, when α (*m* hydroxybenzylidene)- γ methyl Δ^8 γ butenolide (II, $R=H$, $R'=OH$) resulted, under other conditions only a resinous product was formed. Protocatechuic aldehyde gave α (3,4-dihydroxybenzylidene)- γ methyl Δ^8 γ butenolide (II, $R=R'=OH$). Condensation of *o* hydroxyaldehydes did not give compounds of type II, but gave 3 acetonylcoumarins (Marrian & Russell, 1946). The preparation of *o* hydroxyarylidenobutenolides corresponding to (II) was attempted without success by other methods, e.g. preparation of a butenolide in which the *o* hydroxy group was blocked by an acid labile group such as methoxymethyl, followed by acid hydrolysis, failed, although



(I)



(II)



(III)

α (*p* Methoxybenzylidene)- γ methyl Δ^8 γ butenolide (II, $R=OMe$, $R'=H$) was first prepared by Thiele, Tischbein & Lössow (1901) by condensation of anisaldehyde with Δ^8 γ angelicalactone in the presence of a base. Condensations of this type were also carried out by von Oettingen (1930), who prepared a number of compounds including (II, $R=OH$, $R'=OMe$), (II, $R=R'=OMe$) and (II, R and R'

the product obtained by condensation of *p* methoxymethoxybenzaldehyde with Δ^8 γ angelicalactone gave an excellent yield of (II, $R=OH$, $R'=H$) on acid hydrolysis.

p Acetamidobenzaldehyde and Δ^8 γ angelicalactone in the presence of base, with or without solvent, gave α (*p*-acetamidobenzylidene)- γ methyl Δ^8 γ butenolide (II, $R=NHAc$, $R'=H$). *p* Aminobenzaldehyde

in ethanol gave under the same conditions α (p aminobenzylidene) - γ - methyl - Δ^{β} γ - butenolide (II, $R = \text{NH}_2$, $R' = \text{H}$), the constitution of this compound was proved by acetylation with acetic anhydride, which gave (II, $R = \text{NHAc}$, $R' = \text{H}$), identical with the sample prepared as above. With succinic anhydride (II, $R = \text{NH}_2$, $R' = \text{H}$) gave α (p succinamidobenzylidene) - γ - methyl - Δ^{β} γ - butenolide (II, $R = \text{NHCO}(\text{CH}_2)_2\text{COOH}$, $R' = \text{H}$) and with benzene sulphonyl chloride α (p benzenesulphonamidobenzylidene) - γ - methyl Δ^{β} γ - butenolide (II, $R = \text{PhSO}_2\text{NH}$, $R' = \text{H}$) was formed. Two cinnamylidene lactones were also prepared. Despite reports to the contrary (von Oettingen, 1930), cinnamaldehyde condensed with Δ^{β} γ angelicalactone to give α cinnamylidene γ - methyl Δ^{β} γ - butenolide (III, $R = \text{H}$). *p* Hydroxy cinnamaldehyde (Pauly & Wascher, 1923) did not appear to condense, but *p* acetamidocinnamaldehyde, prepared by condensation of acetaldehyde with *p* acetamidobenzaldehyde in presence of potassium hydroxide, gave α (p acetamidocinnamylidene) - γ - methyl - Δ^{β} γ - butenolide (III, $R = \text{NHAc}$).

Other compounds prepared for testing were (II, $R = \text{OH}$, $R' = \text{OMe}$) (von Oettingen, 1930), *p* hydroxybenzylideneacetone (Zincke & Mulhausen, 1903), *o* hydroxybenzylideneacetone (Harries, 1891), *m* hydroxybenzylideneacetone, 3 *o* hydroxybenzylidene 2,4 diketochroman (Sullivan, Huebner, Stahmann, & Link, 1943), β (p hydroxybenzoyl) propionic acid (Swain, Todd & Waring, 1944), methyl-*p*-hydroxycinnamate (Fischer & Nouri, 1917), the amide of *p* hydroxycinnamic acid, 7 hydroxy 4 methylcoumarin (von Pechmann & Cohen, 1884) and 7-hydroxy 3 acetyloumarin (Marrion & Russell, 1946). Attempts were also made to condense 1 acetoxy 4 formylbutadiene 1,4 (glutacondialdehyde enolacetate), 4 hydroxy 1-naphthaldehyde and indole 3 aldehyde with Δ^{β} γ angelicalactone, but no identifiable product was obtained in any of these cases.

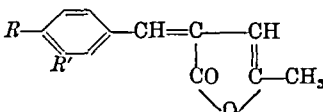
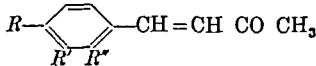
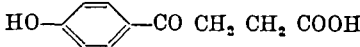
Most of the above compounds were tested as hatching agents for potato eelworm cysts, using the technique already described (Calam, Raistrick & Todd, 1949). The results are shown in Table 1.

In view of the weak activity shown by certain compounds of type (II) it seemed of interest to prepare and test a number of analogously constituted oxazolone derivatives of general formula (IV). For the preparation of such compounds the well-established oxazolone synthesis from aromatic aldehydes and *N* acylglycines was employed. *p* Acetamidobenzaldehyde and hippuric acid gave 2 phenyl 4-(p acetamidobenzylidene) oxazolone 5 (IV, $R = \text{NHAc}$, $R' = \text{Ph}$), with *N* caproylglycine, 2 *n* amyl 4-(p acetamidobenzylidene) oxazolone 5 (IV, $R = \text{NHCOCH}_3$, $R' = n\text{C}_6\text{H}_{11}$) together with α (*N* caproylamino) - *p* - acetamidocinnamic acid (V, $R = \text{NHAc}$, $R' = \text{COC}_6\text{H}_{11}$) resulted. Condensa-

tion of *p* acetamidobenzaldehyde with *N* acetylglycine gave an unidentified red acid $\text{C}_{15}\text{H}_{16}\text{O}_6\text{N}_2$, *m.p.* 246–7°.

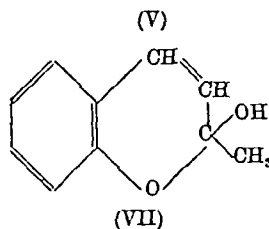
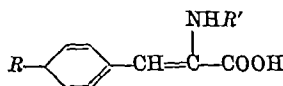
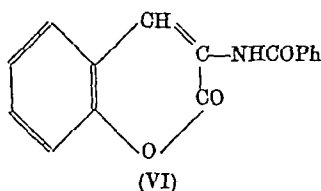
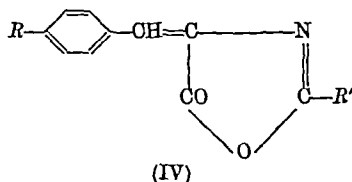
Table 1 Activity of synthetic substances as hatching agents for the potato eelworm

(+, active at concentrations, about 1/5000 to 1/10000, +, active at concentrations, about 1/1000 to 1/5000, \pm , active at concentrations, > 1/5000 but variable hatches obtained, -, inactive)

Substance	Activity
Anhydrotetrone acid	+
	
$R = \text{OH}$, $R' = \text{H}$	++
$R = \text{NHAc}$, $R' = \text{H}$	+
$R = \text{NH}_2$, $R' = \text{H}$	-
$R = \text{OH}$, $R' = \text{OMe}$	++
$R = \text{H}$, $R' = \text{OH}$	-
$R = R' = \text{OH}$	\pm
	
$R = \text{OH}$, $R' = R'' = \text{H}$	\pm
$R = R' = \text{H}$, $R'' = \text{OH}$	-
$R = R'' = \text{H}$, $R' = \text{OH}$	-
	\pm
7 Hydroxy-4 methylcoumarin	-
7 Hydroxy 3 acetyloumarin	-
Methyl <i>p</i> hydroxycinnamate	-
Amide of <i>p</i> hydroxycinnamic acid	-
* 3 (<i>o</i> Hydroxybenzylidene) 2,4 diketochroman	-
* α (p Acetamidocinnamylidene) γ methyl Δ^{β} γ - butenolide	-
* Very low solubility	

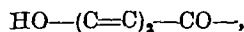
Condensation of the methoxymethyl ether of *p* hydroxybenzaldehyde with hippuric acid gave 2 phenyl 4-(p methoxymethoxybenzylidene) oxazolone 5 (IV, $R = \text{OCH}_2\text{OMe}$, $R' = \text{Ph}$), which was hydrolysed by a little sulphuric acid in 20% aqueous acetic acid to α -benzamido *p* hydroxycinnamic acid (V, $R = \text{OH}$, $R' = \text{COPh}$), apparently identical with Erlenmeyer & Halsey's (1899) preparation. Sublimation of this material *in vacuo* at 200° gave 2 phenyl-4-(p hydroxybenzylidene) oxazolone 5 (IV, $R = \text{OH}$, $R' = \text{Ph}$), a second route to this compound was the hydrolysis of (IV, $R = \text{OCH}_2\text{OMe}$, $R' = \text{Ph}$) with a small amount of H_2SO_4 in glacial acetic acid. The methoxymethyl ether of salicylaldehyde gave 2 phenyl 4-(*o* methoxymethoxybenzylidene) oxazolone 5 with hippuric acid. On hydrolysis with concentrated H_2SO_4 in 20% aqueous acetic acid, the compound gave 3 benzamidocoumarin (VI), identical with a specimen prepared by the method of Erlenmeyer & Stadlin (1904). Condensation of these methoxymethoxybenzaldehydes with *N* acetylgly-

cine gave no identifiable product, although condensation of anisaldehyde and *N* acetylglucosamine gave the expected product (IV, $R = \text{OMe}$, $R' = \text{CH}_3$, cf Sugasawa & Tsuda, 1935). Compounds (IV, $R = \text{NHAc}$, $R' = \text{Ph}$) and (IV, $R = \text{NHAc}$, $R' = n\text{C}_5\text{H}_{11}$) were tested, but were very insoluble and appeared to be inactive (IV, $R = \text{OH}$, $R' = \text{Ph}$) was again very insoluble (1/10,000) in water and showed no activity at this great dilution



The biological test results recorded in Table 1 show that the *p* hydroxy compounds (II, $R = \text{OH}$, $R' = \text{H}$), (II, $R = \text{OH}$, $R' = \text{OMe}$) and (II, $R = R' = \text{OH}$) show definite if weak activity as hatching agents. It is noteworthy that the corresponding *m* hydroxy compound (II, $R = \text{H}$, $R' = \text{OH}$) is quite inactive. *p* Hydroxybenzylideneacetone and β (*p* hydroxybenzoyl) propionic acid also show some activity, while *p* hydroxyacetophenone, which was also tested, showed a doubtful activity. The *p* amino compound (II, $R = \text{NH}_2$, $R' = \text{H}$) was inactive, but replacement of the amino group by the feebly acidic acetamido group as in (II, $R = \text{NHAc}$, $R' = \text{H}$) gave an active substance. Extremely low solubility made the testing of (II, $R = \text{NHCO}(\text{CH}_2)_2\text{COOH}$, $R' = \text{H}$) and (II, $R = \text{NHSO}_2\text{Ph}$, $R' = \text{H}$) impossible. Examination of the structure of the active compounds shows that they contain in common with anhydrotetrone a weakly acidic group joined through a conjugated system to a $\text{C}=\text{O}$ group, i.e. $\text{HO}-(\text{C}=\text{C})_n-\text{CO}-$ or $\text{RCONH}-(\text{C}=\text{C})_n-\text{CO}-$. Such systems when ionized would exist as resonance hybrids, the negative charge making its appearance at both ends of the system—a state of affairs conducive to hydrogen bonding, which may well play a part in attachment of the hatching agent to some specific protein in the eelworm cysts. Whilst this may be a factor in determining whether a compound can display activity, it is evidently not by any means the only one, as can be seen from the comparatively enormous activity of the natural material and its high specificity. Moreover, the lack of activity in

the derivatives of *p* hydroxycinnamic acid and in the oxazolones examined is evidence pointing in the same direction, since these compounds appear to satisfy the requirements as far as the conjugated system goes. The inactivity of *o* hydroxybenzylideneacetone, which contains the system



although at first sight surprising, may be due to the

fact that this compound normally exists in the ψ base form VII (Decker & von Fellenberg, 1909).

In passing, it may be observed that the degree of activity of the compounds may be expressed in a slightly different way from that given in Table 1, anhydrotetrone acid exhibits full hatching activity in water at a dilution of about 1/2000 and α (*p* hydroxybenzylidene)- γ methyl $\Delta^8 \gamma$ butenolide (II, $R = \text{OH}$, $R' = \text{H}$) at a dilution about 1/7500. These activities are much too low for the compounds to have any practical value (purified concentrates of the natural hatching factor show equal activity at dilutions of 1/10⁷), but in view of the high specificity we consider that the results are significant and can form a basis for further synthetic work.

EXPERIMENTAL

α (*p* Hydroxybenzylidene) γ methyl $\Delta^8 \gamma$ butenolide. *p* Hydroxybenzaldehyde (1.2 g, 1 mol) and $\Delta^8 \gamma$ angelicalactone (0.98 g, 1 mol) were mixed together and warmed until liquid. Piperidine (2–3 drops) was added to the mixture and the whole warmed on a steam bath for 15 min. More piperidine (3 drops) was added and the mixture allowed to stand overnight, then dissolved in ether and the solution washed with dilute HCl, water, and dried over Na_2SO_4 . The ether was removed and the product recrystallized from benzene giving deep yellow needles, m.p. 133–134° (Found: C, 71.1, H, 5.3. $\text{C}_{11}\text{H}_{10}\text{O}_2$ requires C, 71.4, H, 5.0%). The substance gives a brick red colour with NaOH, no colour with FeCl_3 and no legal reaction.

α (*m* Hydroxybenzylidene) γ methyl $\Delta^8 \gamma$ butenolide. *m* Hydroxybenzaldehyde (1.2 g, 1 mol) and $\Delta^8 \gamma$ angelicalactone (0.98 g, 1 mol) were dissolved in absolute

ethanol (10 ml) and triethylamine (5–10 drops) added. The mixture was refluxed for 5 hr, the solution cooled and water added until it became opalescent. On allowing to stand crystalline material separated, which on recrystallization from aqueous ethanol gave pale yellow needles, m.p. 134° (Found C, 70.9, H, 5.0. $C_{12}H_{10}O_2$ requires C, 71.3, H, 4.9%). α -(3,4-Dihydroxybenzylidene) γ -methyl Δ^{β} - γ -butenolide. Protocatechuic aldehyde (2.8 g) and Δ^{β} - γ -angelicalactone (1.96 g) were mixed with triethylamine (5 drops), the mixture warmed for 30 min on a steam bath and allowed to stand overnight. The mixture was dissolved in ether and the ethereal solution washed with water and dilute HCl, and finally washed free from acid with water and then dried. The ether was evaporated and the residual yellow solid recrystallized from benzene. The product had no definite crystalline form even under the microscope, but repeated recrystallization from benzene left the melting point unchanged at 135°, analysis indicated that it was probably a complex of the desired product with starting material (Found C, 62.9, H, 5.0. $C_{12}H_{10}O_4 \cdot 2C_7H_6O_2$ requires C, 63.1, H, 4.5%). This material, after several recrystallizations from water, gave the expected lactone as bright yellow needles, m.p. 180° (Found C, 65.9, H, 4.8. $C_{12}H_{10}O_4$ requires C, 66.0, H, 4.6%).

Attempts to prepare α -(o-hydroxybenzylidene) γ -methyl Δ^{β} - γ -butenolide. While condensation of the methoxymethyl ether of *p*-hydroxybenzaldehyde with Δ^{β} - γ -angelicalactone under the above conditions gave an oil which on hydrolysis with dilute H_2SO_4 in 50% acetic acid gave formaldehyde and α -(*p*-hydroxybenzylidene) γ -methyl Δ^{β} - γ -butenolide, m.p. 134°, the condensation product from the methoxymethyl ether of salicylaldehyde and Δ^{β} - γ -angelicalactone gave, on acid hydrolysis under the same conditions, a resinous product from which no pure compound could be isolated.

α -(*p*-Acetamidobenzylidene) γ -methyl Δ^{β} - γ -butenolide. *p*-Acetamidobenzaldehyde (1.65 g, 1 mol.) was mixed with Δ^{β} - γ -angelicalactone (0.98 g, 1 mol.) and piperidine (5 drops) added. The mixture was warmed for 1 hr, allowed to stand for 3 hr, and then triturated with ether and the solid recrystallized from methanol. It gave bright yellow needles, m.p. 196–197° (sintered 185°) (Found C, 69.5, H, 5.8, N, 6.0. $C_{14}H_{13}O_3N$ requires C, 69.3, H, 5.4, N, 5.8%). The condensation could be carried out just as effectively in absolute ethanol (25 ml for above quantities) using triethylamine as catalyst.

α -(*p*-Aminobenzylidene) γ -methyl Δ^{β} - γ -butenolide. *p*-Aminobenzaldehyde (1.2 g, 1 mol.) and Δ^{β} - γ -angelicalactone (0.98 g, 1 mol.) were refluxed in ethanol (10 ml.) with triethylamine (6 drops) for 3 hr. The solution was diluted with water until it became opalescent, on cooling orange needles separated, m.p. 138–139°, after recrystallization from methanol. (Found C, 71.4, H, 5.6, N, 7.1. $C_{12}H_{11}O_2N$ requires C, 71.6, H, 5.5, N, 7.0%). Treatment with acetic anhydride gave α -(*p*-acetamidobenzylidene)- γ -methyl Δ^{β} - γ -butenolide, identical with a sample prepared as above.

α -(*p*-Succinamidobenzylidene) γ -methyl Δ^{β} - γ -butenolide. α -(*p*-Aminobenzylidene) γ -methyl Δ^{β} - γ -butenolide (1.0 g) was well mixed with succinic anhydride (0.5 g), the mixture warmed on a steam bath for 0.5 hr and then allowed to stand overnight at room temperature. The solid was extracted with saturated $NaHCO_3$ solution, the insoluble portion filtered off and the $NaHCO_3$ solution acidified

The precipitate was collected and washed with water, recrystallized from aqueous ethanol it gave brownish orange needles, m.p. 195–100° (Found C, 63.4, H, 4.8, N, 4.7. $C_{16}H_{15}O_5N$ requires C, 63.7, H, 5.0, N, 4.7%). The $NaHCO_3$ insoluble portion was found to be unchanged α -(*p*-amino benzylidene) γ -methyl Δ^{β} - γ -butenolide.

α -(*p*-Benzenesulphonamidobenzylidene) γ -methyl Δ^{β} - γ -butenolide. α -(*p*-aminobenzylidene) γ -methyl Δ^{β} - γ -butenolide (1.0 g), benzenesulphonyl chloride (0.8 ml) and pyridine (5 ml) were warmed on a steam bath for 30 min. The mixture was poured on ice (30 g), the solid washed with $NaHCO_3$ solution and recrystallized from ethanol giving yellow needles, m.p. 200–201° (Found C, 63.1, H, 4.4, N, 4.2. $C_{18}H_{15}O_4NS$ requires C, 63.4, H, 4.4, N, 4.1%).

α -Cinnamylidene γ -methyl Δ^{β} - γ -butenolide. Cinnamaldehyde (1.3 g) and Δ^{β} - γ -angelicalactone (0.98 g) were mixed together with triethylamine (3–4 drops) heated for 1 hr and then allowed to stand overnight. The solid which separated was filtered off and washed with ether, recrystallized from ethanol it gave orange yellow needles, m.p. 139–140° (Found C, 79.0, H, 5.8. $C_{14}H_{12}O_2$ requires C, 78.8, H, 5.7%).

p-Acetamidocinnamaldehyde. A solution of acetaldehyde (0.4 ml.) in ethanol (15 ml.) was slowly added to an ice cold stirred solution of *p*-acetamidobenzaldehyde (1.0 g), and ethanolic KOH (30 ml, 2%). Stirring was continued at 0° for a further 3 hr, the mixture exactly neutralized, evaporated *in vacuo* and the residue extracted thoroughly with ether. The extract was evaporated and the residual red oil dissolved in water (120 ml, with charcoal), filtered and set aside overnight. The solid which separated was recrystallized several times from water giving deep yellow needles, m.p. 175–176° (decomp). (Found C, 69.7, H, 5.8, N, 7.7. $C_{11}H_{11}O_2N$ requires C, 69.8, H, 5.8, N, 7.4%). The substance gave a semicarbazone, m.p. 229° (decomp) (Found N, 22.3. $C_{11}H_{11}O_2N_2$ requires N, 22.8%).

α -(*p*-Acetamidocinnamylidene) γ -methyl Δ^{β} - γ -butenolide. *p*-Acetamidocinnamaldehyde (0.65 g) and Δ^{β} - γ -angelicalactone (0.31 g) were mixed together and triethylamine (3–4 drops) added. The mixture was heated on a steam bath for 15 min and allowed to stand overnight at room temperature. The semi-crystalline mass which separated was triturated with methanol giving orange prisms, m.p. 203–206°. On recrystallization from the same solvent it had m.p. 204–205° (softens 195°) (Found C, 71.7, H, 5.4, N, 5.2. $C_{16}H_{15}O_3N$ requires C, 71.4, H, 5.6, N, 5.2%).

m-Hydroxybenzylidenacetone. *m*-Hydroxybenzaldehyde (5 g), acetone (20 ml) and KOH (14 ml of 10%) were left to stand at room temperature for 3 days. The solution was acidified with HCl and the oil extracted with ether. After washing with water and drying, the ether was evaporated and the residue recrystallized from benzene giving yellow prisms, m.p. 95–96° (Found C, 74.3, H, 6.3. $C_{10}H_{10}O_2$ requires C, 74.1, H, 6.2%).

2-Phenyl 4-(*p*-acetamidobenzylidene) oxazolone. 5-*p*-Acetamidobenzaldehyde (3.26 g), hippuric acid (3.58 g), anhydrous sodium acetate (1.68 g) and acetic anhydride (5.1 g) were heated together on a steam bath for 45 min. The mixture was cooled and the orange solid triturated with ethanol, filtered and recrystallized from acetic acid, yellow prisms, m.p. 237–239° (Found C, 70.4, H, 4.7, N, 9.2. $C_{18}H_{14}O_2N_2$ requires C, 70.6, H, 4.6, N, 9.2%).

2-n-Amyl 4-(*p*-acetamidobenzylidene) oxazolone. 5-N-Caproylglycine (3.0 g) and *p*-acetamidobenzaldehyde

(3.0 g) were condensed as described above and the resulting solid on recrystallization from methanol gave deep yellow prisms, m p 141–142° (Found C, 68.2, H, 6.3, N, 9.4 $C_{17}H_{20}O_2N$ requires C, 68.0, H, 6.6, N, 9.3%) The mother liquors on dilution with water gave a substance which crystallized in pale yellow needles, after recrystallization from aqueous methanol it had m p 225–226° (decomp) (Found C, 64.5, H, 7.0, N, 9.1 $C_{17}H_{22}O_4N_2$ requires C, 64.3, H, 6.9, N, 9.2%) This compound was α -n-caproylamino p-acetamidocinnamic acid.

Condensation of acetyl glycine with p-acetamidobenzaldehyde Acetyl glycine and p-acetamidobenzaldehyde were condensed together in the usual manner. The product formed orange red prisms, m p 246–247° from water. After drying *in vacuo* it gave the following analysis (Found C, 55.6, H, 5.9, N, 10.0, Equiv, 302 $C_{15}H_{16}O_5N_2$ requires C, 55.7, H, 5.7, N, 10.0%, Equiv, 280).

2 Phenyl 4 (o-methoxymethoxybenzylidene) oxazolone 5 Salicylaldehyde methoxymethyl ether (3.32 g, Hoering & Baum, 1909), and hippuric acid (3.58 g) were condensed together in the usual manner. The product crystallized from acetic acid in yellow needles, m p 139–140° (Found C, 69.6, H, 5.0, N, 4.6 $C_{18}H_{15}O_4N$ requires C, 69.8, H, 4.9, N, 4.5%)

This material (0.8 g) was heated on a steam bath for 1.5 hr with aqueous acetic acid (10 ml, 20%) and H_2SO_4 (4 drops), formaldehyde was evolved. On cooling colourless needles separated, m p 175–176°, undepressed in admixture with 3-benzamudocoumarin prepared by the method of Erlenmeyer & Stadlin (1904).

2 Phenyl 4 (p-methoxymethoxybenzylidene) oxazolone 5 This was prepared as above from p-methoxymethoxy benzaldehyde (Pauly & Wäscher, 1923) and hippuric acid, bright yellow needles from glacial acetic acid, m p 122–123° (Found C, 70.1, H, 5.1, N, 4.9 $C_{18}H_{15}O_4N$ requires C, 69.8, H, 4.9, N, 4.5%)

α -Benzamido p-hydroxycinnamic acid The above azlactone (0.5 g) was hydrolysed with aqueous acetic acid (20 ml, 20%) and H_2SO_4 (4 drops) on a steam bath for 1 hr, by which time no more formaldehyde was evolved. On cooling, colourless prisms separated which on recrystallization from acetic acid had m p 227–228° (decomp), turning yellow at about 200° (Erlenmeyer & Halsey (1899) give m p 228–229°).

2 Phenyl 4-(p-hydroxybenzylidene) oxazolone 5 The above acid was sublimed in a high vacuum at 200° yielding a yellow solid m p 220°, undepressed in admixture with a sample prepared by method (u) below.

(u) 2 Phenyl 4 (p-methoxymethoxybenzylidene) oxazolone-5 (1.0 g) was heated to boiling with acetic acid (2 ml) containing a trace of conc H_2SO_4 . On cooling, a yellow solid separated which, when purified on an alumina column followed by vacuum sublimation at 160°/10⁻⁶ mm, had m p 220–221° (Found C, 72.0, H, 4.2, N, 5.2 $C_{18}H_{15}O_4N$ requires C, 72.5, H, 4.2, N, 5.3%)

SUMMARY

1 A series of compounds has been prepared based on the α -arylidenes Δ^8 γ -butenolide skeleton, and containing conjugated systems analogous to that present in anhydrotetrone acid. Several of the synthetic products show definite, if rather low, activity as hatching agents for the potato eelworm. The structural similarity between the active substances is discussed.

2 No hatching activity was detected in a number of coumarin and oxazolone derivatives.

The authors are indebted to the Agricultural Research Council for grants in aid of this investigation.

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The Fermentation Process in Tea Manufacture

10 THE CONDENSATION OF CATECHINS AND ITS RELATION TO THE CHEMICAL CHANGES IN FERMENTATION

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(Received 11 April 1940)

In an earlier communication in this series, Harrison & Roberts (1939) outlined what was known, at the time, of the chemistry of the so called tea tannins. More recently Bradfield (1946) and Bradfield, Penney & Wright (1947) have examined the polyphenols in tea by the method of partition chromatography and have established that all the components investigated are derived either from epicatechin or galocatechin. In view of this positive identification it seems advantageous to refer in future to the polyphenols in tea leaf as catechins.

The reasons for believing that catechins undergo condensation during fermentation after oxidation were summarized by Harrison & Roberts (1939), and further evidence for this belief was afforded by a comparison of the Löwenthal and alkaline iodine titration methods with the Stamm procedure, as applied to tea catechins by Barua & Roberts (1940). Reference to the work of Lamb & Sreerangachar (1940) and Bradfield & Penney (1944) will show that other workers share this view.

The present communication describes experiments, carried out mainly in 1940 and 1941, in which an attempt was made to devise some means of estimating the extent of condensation and to relate it to other chemical changes in fermented tea leaf. A brief outline of these findings has already been presented (Roberts, 1942).

Teas were fermented at temperatures of 60, 70, 80 and 90° F, varying the time of fermentation from 2 to 5.75 hr. Water soluble solids, water soluble catechins and alcohol soluble (i.e. ethanol soluble) solids were determined on these teas, and the changes in these figures, due to different extents of fermentation, related to the degree of condensation. Less extensive data relating the amount of extractable caffeine to the degree of fermentation are also presented.

Correlations were also found between the extent of condensation and the development of certain liquor characters recognized by the tea taster. These findings will be reported elsewhere.

METHODS

Manufacture of teas

Under normal conditions of manufacture the tea leaf undergoes mechanical rolling for some considerable time, during which period temperature control cannot be maintained. In order to carry out fermentation as far as possible under controlled conditions the period of rolling was reduced to 30 min, after which the leaf was rapidly passed through a McKercher C T C machine. This machine is sometimes used in commercial factories to bring about further bruising of the coarser pieces of tea leaf after rolling is completed. In our experiments it was used to complete the damage necessary for fermentation within a reasonably short time. The remainder of the fermentation was carried out in air conditioned cabinets at one of four temperatures, 60, 70, 80, or 90° F, the relative humidity was 95–100%. After 2, 2.75, 3.5, 4.25, 5 and 5.75 hr fermentation (taken from the time at which rolling was started) samples of leaf were dried at 180° F.

In this way teas which had received twenty four different fermentation treatments were prepared. The series was repeated eight times in the manufacturing season of 1940. As only two cabinets were available, only two temperatures of fermentation could be dealt with in 1 day. All teas were manufactured from one block of tea, plucked at weekly intervals, so that 2 separate weeks' pluckings were required to cover all four temperatures of fermentation. Temperatures were randomized so that in the complete series of manufactures, lasting 16 weeks, no one temperature could receive bias owing to seasonal trends.

In all, therefore, 192 different teas were available for investigation, but it was usual to obtain highly significant results with fewer samples.

Analytical procedures

The condensation index. The exact determination of the extent of condensation of catechins is hampered by lack of knowledge, and measurements must therefore be largely empirical. According to Harrison & Roberts (1939) the catechins precipitated from a tea infusion by 1% (v/v) H_2SO_4 probably represent the most highly condensed fraction. Advantage has been taken of this observation and the following procedure adopted.

A portion (20 ml) of an infusion, obtained by refluxing 5 g tea with 400 ml water for 1 hr and making up to

500 ml, is treated with 0.2 ml conc H_2SO_4 . After standing for 1 hr the precipitate obtained is centrifuged down, washed once with 1% (v/v) H_2SO_4 and suspended in water. The suspension is then titrated according to Stamm's procedure (Barua & Roberts, 1940) and the result expressed as ml 0.1M $KMnO_4$ /g of made tea (dry wt). The titre is a measure of the catechins precipitated by 1% H_2SO_4 and is referred to subsequently as the condensation index or C.I. The method must be considered purely tentative, justified at present because it gives semi quantitative and intelligible results.

Water-soluble catechins were determined by the method of Barua & Roberts (1940), which is applicable to the soluble catechins in green leaf and fermented tea.

Water insoluble catechins The spent tea leaf, after extraction to make up the 1 hr infusion, is extracted a second time for 1 hr with 500 ml water. The residue from the second extract is washed with boiling water, dried and weighed. The dried residue (50 mg) is suspended in 2 ml water in a Warburg vessel, and 1 ml 0.2N NaOH tipped in after attainment of temperature equilibrium. The total O_2 uptake, obtained after shaking for 2 hr, expressed as $\mu l O_2$ /mg dry wt of the original tea, is a measure of the water insoluble catechins. It may be compared with the figure obtained for the 1 hr infusion similarly expressed as $\mu l O_2$ /mg dry wt of made tea.

Caffeine A 1 hr or 5 min infusion (200 ml) is made alkaline with 10 ml ammonia (sp gr 0.880). The solution is then extracted six times with 50 ml portions of $CHCl_3$, the solvent removed from the united extracts by distillation and the weight of the residue taken as caffeine.

Oxygen uptake during fermentation To determine the O_2 uptake which has occurred at any stage of fermentation, the partly fermented leaf is very finely minced and its total uptake determined manometrically. Subtraction of this uptake from the total uptake of a sample of the unfermented green leaf gives the uptake up to the time of sampling (cf Roberts, 1941).

RESULTS

Variation of condensation index with time and temperature of fermentation The condensation index of all 192 teas was determined. On three occasions, however, as a result of high withers, in which the moisture content of the tea leaf fell in 18 hr to about 50%, instead of the normal 65–70%, the teas fermented rather more slowly. These three sets have been left out in calculating the mean values for the whole season, which are recorded in Table 1. The increases in the condensation index with the time and temperature of fermentation are almost linear.

Table 1 Effect of time and temperature of fermentation on the condensation index

(Values are condensation indices)

Temp (°F)	Fermentation time (hr)					
	2	2.75	3.5	4.25	5	5.75
60	24.3	28.6	32.7	36.8	40.0	43.3
70	27.3	32.1	37.3	42.6	46.9	51.1
80	27.6	34.6	41.2	47.7	53.4	59.1
90	33.4	40.2	47.8	55.8	60.3	64.8

For comparison, the O_2 uptake at different stages of fermentation is recorded in Table 2. Each figure is a mean of three separate manufactures.

Table 2 Oxygen consumption during fermentation

Temp (°F)	Fermentation time (hr)		
	2	4	5.75
	(Total $\mu l O_2$ /mg dry wt)		
60	6.8	8.4	9.6
70	7.6	9.5	10.0
80	8.3	10.4	11.0
90	8.2	10.3	10.8

The results indicate a clear cut distinction between oxidation and condensation of the catechins. Between 2 and 4.25 hr fermentation at all temperatures the condensation index increases linearly, with some slight falling off from 4.25 to 5.75 hr, whereas the rate of O_2 uptake decreases as fermentation proceeds. The condensation index increases proportionately with temperature, but the increase in rate of O_2 uptake with temperature is not maintained beyond 80°F.

It would appear logical to conclude that although condensation follows oxidation, the subsequent rate of condensation is independent of the rate of oxidation, and is determined largely by time and temperature.

Water soluble solids and catechins It has long been known that the water soluble solids decrease progressively as a result of fermentation. In Table 3 the mean values are given for water soluble solids, water soluble catechins and condensation indices for four times and four temperatures of fermentation. Values for five series of manufacture only are given, as the three high withered teas were omitted.

As with the condensation index, variations in soluble solids and catechin with time and temperature of fermentation are very highly significant. Correlations between any two of these three variables are also found to be significant, but the eighty pairs of data cannot be considered homogeneous, owing to the systematic differences due to time, temperature and date of manufacture. It is therefore necessary to calculate the correlation coefficient (r) for each factor using the analysis of covariance.

The changes in soluble solids and catechins, due to varying time and temperature of fermentation, are significantly correlated with the condensation index, and with each other (Table 4). The only other significant effect is the residual correlation between soluble solids and catechins. As the catechins account for nearly half the total water soluble solids, it is not unexpected that such a correlation should be found.

Table 3 *Effect of time and temperature of fermentation on soluble solids, soluble catechins and condensation index*

(C.I. = condensation index.)

Time (hr)		2			3 5				4 25			5 75			
Temp (° F)		Soluble solids (%)	Cate chin (%)	C.I.	Soluble solids (%)	Cate chin (%)	C.I.		Soluble solids (%)	Cate chin (%)	C.I.	Soluble solids (%)	Cate chin (%)	C.I.	
60	Mean	43.2	19.2	26.2	42.1	18.4	35.9		41.8	17.6	40.9	41.1	17.7	48.5	
	S.E.	0.25	0.56	1.8	0.51	0.37	2.4		0.51	0.40	2.6	0.47	0.34	3.4	
70	Mean	42.3	18.5	27.1	41.2	17.9	38.3		39.9	17.4	44.1	39.7	17.2	52.7	
	S.E.	0.78	0.46	1.8	0.91	0.62	1.0		0.74	0.49	1.4	0.73	0.35	1.8	
80	Mean	42.0	18.0	30.5	39.7	17.1	46.2		38.5	16.7	54.1	38.3	15.8	63.0	
	S.E.	0.35	0.71	0.8	0.57	0.62	1.9		0.72	0.81	2.7	0.99	0.60	0.9	
90	Mean	41.1	17.1	34.3	39.4	16.0	49.1		39.1	15.7	57.2	38.5	15.1	65.6	
	S.E.	0.56	0.55	3.1	0.38	0.53	3.1		0.45	0.70	3.3	0.64	0.40	2.7	

Table 4 *Analysis of covariance Soluble solids, soluble catechins and condensation index (C.I.)*

	Degrees of freedom	Soluble solids and catechins	Soluble solids and C.I.	Catechins and C.I.
Temperature	3	0.903*	-0.906*	-0.978†
Time	3	0.993†	-0.969†	-0.997†
Date	4	-0.038	0.167	0.168
Temperature × time	9	0.143	-0.279	-0.176
Temperature × date	12	0.312	-0.254	-0.231
Time × date	12	0.304	0.516	0.298
Residual	35	0.392*	-0.139	-0.194

* $P=0.05\%$ † $P=0.01\%$ ‡ $P=0.001\%$

The correlation coefficient (r) between all eighty pairs of data for soluble solids and catechins is 0.5649. If the date variance and the interactions with date are eliminated by averaging each set of five figures, we are left with sixteen comparisons, for which r now becomes 0.9093. A straight line relation may be calculated from these figures (Catechin %) = $-9.6106 + 0.6624$ (soluble solids %).

For a fall of 1.0% in the water soluble solids there is a corresponding decrease of 0.66% in the catechins. Thus, although the decreasing solubility in water of the catechins is a major factor in the decrease in water soluble solids as a result of fermentation, it is probable that other water soluble substances are also concerned. This could, perhaps, be accounted for by an oxidation of respiratory substrates to CO_2 by the orthoquinones formed on oxidation of the catechins, or by a combination of oxidized and condensed products with water soluble nitrogenous substances to form an insoluble product.

Water insoluble catechins Table 5 gives average figures of oxygen uptake determined in 1939 for water soluble and insoluble catechins, obtained by the method of alkaline autooxidation, for teas fermented for 2.5 and 4.75 hr at 60 and 90° F. The averages are of duplicate experiments in good agreement. The fall in water soluble catechins is accompanied by a distinct increase in water-insoluble catechins.

Table 5 *Alkaline autooxidation of soluble and insoluble catechins in made tea*

		Fermentation time (hr)	
Temp (° F)		2.5	4.75
		$\mu\text{L O}_2/\text{mg}$ original tea	dry wt
Soluble	60	42.7	41.0
	90	38.9	34.4
Insoluble	60	9.9	11.7
	90	12.2	13.0

Caffeine Soluble solids and catechins in the 1 hr infusion both decrease appreciably as a result of fermentation. The fall in soluble solids cannot entirely be accounted for in terms of loss of soluble catechins, and in the previous section it was pointed out that combination of oxidized catechins with soluble nitrogenous substances to form a water insoluble product was a possibility.

The results in Table 6 show that with increase in the time or temperature of fermentation there is no detectable effect on the caffeine content in either the 1 hr or 5 min infusion. These results prove that during fermentation the water insoluble products are not produced by combination with caffeine. It might also be expected that, as a result of the known combination of catechins with caffeine, there would be an appreciably lowered extractability of caffeine in the taster's 5 min infusion, but the results in Table 6 disprove this expectation also.

Table 6 *Effect of fermentation on water soluble caffeine*

Fermentation		Caffeine content (%)		Condensation index
Time (hr)	Temp (° F)	5 min infusion	1 hr infusion	
2	60	2.97	4.30	21.0
2.75	60	3.17	4.40	24.0
4.25	60	2.82	4.50	26.5
4.25	60	3.02	4.50	32.0
5.75	60	3.00	4.35	39.0
4.25	70	2.97	4.50	36.5
4.25	80	2.94	4.35	38.0
2	90	2.97	4.30	32.2
2.75	90	2.92	4.40	37.5
4.25	90	2.94	4.55	58.2
4.25	90	3.25	4.60	47.2
5.75	90	2.90	4.30	66.8

Alcohol-soluble solids As first established in this laboratory in 1930 (unpublished observations), and independently in Indo China by Castagnol & Doan ba Phuong (1940), alcohol extracts much less material than water from fermented tea. According to the latter authors the percentage of alcohol soluble solids decreased with increase in fermentation time and the amount of soluble matter, removed in a subsequent extraction of the residue with water, increased, estimation of the catechins by the hide powder method (Association of Official Agricultural Chemists, 1945) showed that, with increasing time of fermentation, the proportion of catechins fell in the alcohol extract, and increased in the subsequent aqueous extract. The hide powder method has been found in this laboratory (unpublished observations) to give uncertain results with made tea infusions.

Table 7 *Variation in alcohol and water-soluble solids*

(A.S.S. = alcohol soluble solids (%), W.S.S. = water soluble solids (%), C.I. = condensation index)

Fermentation		A.S.S.	W.S.S.	W.S.S. - A.S.S.	C.I.
Time (hr)	Temp (° F)				
2	60	37.5	44.0	6.5	27.5
2	60	37.0	43.3	6.3	25.5
2	60	37.2	42.8	5.6	25.0
5.75	60	33.2	40.6	7.4	48.0
5.75	60	33.1	42.0	8.9	44.5
3.5	70	33.9	42.5	8.6	37.1
5.75	70	30.6	40.8	10.2	52.8
3.5	80	31.1	38.1	7.0	39.3
2	90	37.7	—	—	24.5
2	90	33.1	39.2	6.1	33.5
4.25	90	27.6	39.5	11.9	56.8
5.75	90	25.6	36.9	11.3	66.8
5.75	90	25.7	37.8	12.1	64.8

Table 7 records values obtained in this laboratory for alcohol soluble solids (A.S.S.), water soluble products (W.S.S.) and condensation index (C.I.). The regression coefficients of A.S.S. on C.I., and (W.S.S. - A.S.S.) on C.I. are highly significant, the values for t_{11}

and t_{10} respectively amounting to 11.13 and 6.90, for both of which P is < 0.001 .

The observations of Castagnol & Doan ba Phuong (1940) relating to the extractability of catechins by alcohol are also confirmed, as the catechin content of the aqueous extract of the residue, after alcohol extraction, amounted to 3.79% for a tea fermented 2 hr at 60° F and 6.81% for a tea fermented 5.75 hr at 90° F.

Scarcity of solvents made it impossible to extend these observations to caffeine at the time. Recently, however, it has been established that with increasing fermentation the extractability of caffeine in alcohol also decreases. In teas fermented for 2 and 5 hr at 85° F, 0.41 and 0.60% caffeine respectively (on dry wt) has been found in the aqueous extracts of the residue left after extraction with alcohol.

DISCUSSION

Condensation of the catechins could account for changes in chemical composition and in liquor characters, which cannot be interpreted in terms of oxidation alone. An increase in the fermentation time from 3 to 4 hr in one series of factory experiments, increased the oxidation of catechins from 77 to 80%. The changes in water soluble solids, in depth of colour of infusion and in the strength of the liquor, as estimated by a tea taster, were however quite incommensurate with this slight increase in oxidation. The effect of the extra fermentation would be to increase the condensation index from about 40 to 50. If this figure is related to the actual degree of condensation in a linear manner, this represents an increase of 25% in the condensation, as compared with 3-4% in the oxidation.

Apart from the obvious variations of liquor characters with time and temperature of fermentation, many of the chemical changes observed can be related to the condensation index. There is, in particular, a very close connexion between this value and the total amount of catechin in solution, increasing condensation resulting in decreased water-solubility of the catechins. This may in part be due to the formation of high polymers of catechins insoluble in water, but judging by the increased ability of the catechins to precipitate gelatine as fermentation proceeds, it appears that this decrease must also be partly due to an actual combination of catechin condensation products with leaf protein to form a water insoluble product.

The presence of water insoluble polyphenols in fermented tea has been demonstrated by the method of alkaline auto-oxidation. These insoluble substances increase with fermentation, but can only be estimated in an empirical way.

The existence of highly polymerized water-insoluble catechins, whether combined with protein

or not, accounts in part for the persistence of the brown colour of spent tea leaf after repeated extractions with water. Oxidation products of chlorophyll, according to Sreerangachar (1943), may also be responsible for the development of a brown pigment in the leaf on fermentation, but the suggestion of Popatov (1932) that these pigments are melanins obtained by oxidation of tyrosine cannot be accepted, as not only is the tyrosine content of tea leaf very small, but tyrosine is not oxidized by either tea oxidase or tea oxidase + catechins (unpublished observations).

The figures available for caffeine in the 1 hr infusion suggest that there can be little or no combination between catechin condensation products and caffeine to form substances insoluble in boiling water. The values for alcohol soluble solids suggest, however, that there is an increasing degree of combination between catechins and caffeine as the condensation index increases, with formation of products which are soluble in boiling water but not in boiling alcohol.

Infusions of tea in boiling water are initially clear, but on cooling frequently become cloudy owing to the separation of a finely divided precipitate containing both catechins and caffeine (cf Bradfield & Penney, 1944). It has always been observed in this laboratory that this effect, known as *creaming down*, increases with both time and temperature of fermentation. This is a further indication that combination between catechins and caffeine increases with the extent of condensation of the former. Such combination does not decrease the extraction of soluble matter by

boiling water, but solubility in cold water and alcohol is appreciably reduced.

SUMMARY

1 An empirical method is described for determining the degree of condensation of catechins in fermented teas.

2 The condensation index increases in approximately linear fashion with time and temperature of fermentation, this increase continues at times and temperatures when the total oxygen uptake is no longer increasing.

3 Values for water soluble solids and water soluble catechins, in teas receiving varying fermentation, are significantly correlated with each other, and are correlated negatively with the condensation index. About two thirds of the loss in soluble solids can be accounted for by an insolubilization of catechins, due to the formation of high polymers in soluble in water, and by combination of condensation products with leaf protein.

4 The much greater fall in alcohol soluble solids with increase in the condensation index indicates a combination of condensation products with water soluble nitrogenous compounds to form complexes soluble in boiling water, but insoluble in boiling alcohol.

The author wishes to express his thanks to Mr C J Harrison, Chief Scientific Officer, for his interest in this work and to the Indian Tea Association for permission to publish these results.

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The Deamination of Glycine by α -Radiation from the Disintegration of Boron in a Nuclear Reactor

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(Received 28 June 1949)

A correct assessment of the effects of α radiation as an example of densely ionizing radiations, in comparison with a less densely ionizing radiation (e.g. X-rays), on a variety of solutes in aqueous solutions, is of importance for an understanding of the mode of action. There are a few such comparative investigations reported in the literature (Dale, Gray & Meredith, 1949, this paper contains references to relevant literature). In general α radiation is less effective than X radiation. In some of these investigations it is doubtful whether there was a sufficient consideration of the part played by simultaneous β and γ radiation and, therefore, the small effect observed may have been entirely due to radiation other than α rays. Such effects were fully taken into account in a recent investigation on a comparison between α - and X radiation effects on carboxypeptidase (Dale, Gray & Meredith, 1949). It was also shown that an increase of the concentration of the solute led to an increase of the α ray effect, and the authors inclined to the view that the effects observed were due to the δ rays issuing from the α ray track. Radiation from a polonium source would entirely avoid interference by β or γ radiation, but it is experimentally difficult to make sufficiently strong sources of radiation. This paper reports an investigation of the effects of α -radiation using the disintegration particles from the action of slow neutrons on a boron salt present in the solution of the solute to be investigated. The advantage of this procedure is that not only a large radiation dose can easily be obtained using the neutron flux available in nuclear reactors, but also that the yield from the interfering β and γ radiation can be directly measured.

The reaction investigated was the deamination of glycine by radiation, and this reaction is of interest for several reasons. Glycine has a low molecular weight (75) in contrast to the high molecular weight of carboxypeptidase (35,000), furthermore, solutions of higher concentrations than are possible with carboxypeptidase can be made, and finally the deamination has been shown (Dale & Davies, 1949, Dale, Davies & Gilbert, 1949) to have an ionic yield for X rays rising to nearly 3 on increasing the concentration of glycine to 20%, whereas the X ray ionic yield with carboxypeptidase remains constant at 0.19 over a very wide range of concentrations.

EXPERIMENTAL

Dosimetry

The exposure to slow neutrons of a solution containing a B salt produces an irradiation of the solution by α particles and slow L_1 particles resulting from the nuclear reaction $^{10}\text{B}(\text{n}\alpha)^7\text{Li}$. With a neutron flux of $10^{10}/\text{cm}^2/\text{sec}$ or more, available in nuclear reactors, one can obtain in a few hours sufficient radiation from the heavy particles to give good yields of NH_3 from the deamination of glycine. However, in a nuclear reactor there is also a considerable flux of fast neutrons and γ radiation and these will produce further deamination via their corresponding secondary radiations, protons and electrons. There is also the slow neutron reaction $^{14}\text{N}(\text{n}\text{p})^{14}\text{C}$ originating from the nitrogen in the glycine. These effects can be eliminated by exposing two solutions of the same glycine concentration side by side in the neutron flux, one containing a B salt and one without, the difference in the yields being due to the heavy particle radiation only. We, in fact, irradiated a third solution containing double the amount of B salt, to check that the deamination by the α particles was proportional to the B concentration. For reliable results the deamination in presence of B should be as large as possible compared with the background effect, and this is achieved by using the highest possible concentration of B. It was found by preliminary experiments that up to 2.25M KBO_3 (potassium metaborate) could be added to the glycine solution without affecting the deamination yields by X radiation. The resulting solution was, however, strongly alkaline, and accordingly special experimental procedure had to be adopted, as discussed later. Little prior information about the intensity of the γ radiation in the nuclear reactor was available, but rough estimates indicated that this amount of B would be sufficient, as was finally shown by the experiment.

The rate of liberation of energy/unit volume of solution by the B disintegration can be calculated from the B concentration, the disintegration cross section for thermal neutrons and the average neutron flux in the solution. The mean energy release in the slow neutron disintegration of B can be calculated from the masses (Mattauch & Flüge, 1942), remembering that only about 8% of the disintegrations liberate the full energy, the remainder leaving the ^7Li in an excited level of 474 keV (Boggild, 1945, Gilbert, 1948, Rosenfeld, 1948). These calculations give a mean energy/disintegration of 2.49 MeV. The effect of the 474 keV γ radiation resulting from the de excitation of the ^7Li will not be eliminated by comparison with the B free solution but the solution was in a layer of only 2 mm thickness and absorbs a negligible amount of the γ radiation. The

capture cross section for the B disintegration is taken as $703 \times 10^{-24} \text{ cm}^2$, and therefore the rate of energy liberation/ml of molar solution of B for a neutron flux $n/\text{cm}^2/\text{sec}$ is

$$6.06 \times 10^{20} \times 703 \times 10^{-24} \times 2.49 \times 10^6 n = 1.06 \times 10^6 n \text{ eV/sec}$$

For the purpose of calculating the ionic yield we can define an ion pair as the absorption of 32.5 eV and hence the rate of production of ion pairs/ml of M borate solution due to the α and Li particles is 3.26×10^4 n/sec

For the determination of n , the average neutron flux, account must be taken of the considerable local disturbance of the neutron flux produced by the presence of so much B, and also of the fact that the flux will vary markedly through out the solution. These effects can be allowed for by using the presence of K as a monitor, through the activity of the ^{42}K produced by the slow neutron reaction $^{41}\text{K}(n, \gamma)^{42}\text{K}$, for the number of ^{42}K atoms produced is proportional to the number of B disintegrations independent of the spatial variations of the neutron flux. All solutions were therefore made to have nominally the same K concentration, the appropriate amount of K_2CO_3 being added to the solutions with less or no KBO_2 .

Since the half life of ^{42}K is 12.4 hr and the irradiation of the B solution was 20.9 hr allowance was made for the decay of the ^{42}K during irradiation in calculating the integrated dose. A similar correction for decay between the time of irradiation and measurement was also made. The activity of a K sample was followed for 72 hr to check the half life and a value of 12.8 hr was obtained. This is higher than the generally accepted value of 12.44 (Seaborg & Perlman, 1948) by more than the statistical errors. The measured value was used in the corrections. If this higher value were due to an impurity, its adoption gives the smaller error.

The K monitor was calibrated by two methods. In the first, some samples of 1.48M K_2CO_3 solution were irradiated for several days in the nuclear reactor in positions where the neutron flux had been calibrated. The second method involved calibrating the efficiency of a Geiger counter for ^{42}K and using the activation cross section for ^{41}K (Seren, Friedlander & Turkel, 1947). The counter was of the Veall type (Veall, 1948) and was calibrated with a ^{42}K solution which had been measured at the Atomic Energy Research Establishment, Harwell, in a counter of known efficiency. The two methods give results which differ by about 14%, the first method giving the lower value of the neutron flux. This difference probably indicates the order of magnitude of the error in determining the absolute value of the neutron flux. The mean of the two methods was adopted in the calculations. The neutron flux determined by this method for the B free solutions compared well with the estimated value for their positions in the nuclear reactor.

Chemical method

The general chemical procedure for estimating the NH_3 liberated in the deamination by radiation was the same as in previous work (Dale, Davies & Gilbert, 1949), but since the highly concentrated solution of KBO_2 required could not be neutralized without precipitation it was necessary to carry out the irradiation in the Conway units themselves to prevent loss of NH_3 . The standard pyrex or soda glass

Conway units are not suitable for irradiation inside a nuclear reactor because of the boron and sodium content of the glasses, which either absorb neutrons too strongly or are too radioactive after irradiation. Perspex was therefore used, and sets of three units were hollowed out of solid blocks $6 \times 2 \times 1$ in with perspex sheets ground to form covering lids, which were held in place by clips. Each block was packed into an aluminium can with absorbent cotton wool for insertion into the nuclear reactor. The three Conway units in each block had inner compartments and outer rings which were filled with 1 ml of fluid to a depth of about 2 mm. An 0.25N H_2SO_4 solution was put in all inner compartments. The outer rings contained the glycine solution with varying amounts of KBO_2 and K_2CO_3 , the glycine content being the same in the three units of any one block. The same pattern of KBO_2 and K_2CO_3 concentration was repeated in all blocks and is shown in Table 1.

Two glycine concentrations were investigated, 200,000 and 10,000 $\mu\text{g/ml}$ made up by weight, the higher concentration being duplicated. In all, three perspex blocks were irradiated for 20.9 hr in a neutron flux of about $10^{19}/\text{cm}^2/\text{sec}$ in the nuclear reactor GLEEP at the Atomic Energy Research Establishment, and after removal from the reactor the units were rocked for 0.5 hr to ensure the completion of the diffusion of NH_3 into the acid solution. The solutions from all compartments in the Conway dishes were then transferred quantitatively to test tubes and the subsequent measurements made in our laboratory in Manchester.

Blank experiments to determine the yield of NH_3 in the absence of radiation were made at the same temperature (31°) experienced during the irradiation, and for the same duration, and also control experiments under the same conditions but with added H_2O_2 . According to Bonet-Maury & Lefort (1948) H_2O_2 is formed in pure water proportionately to the α ray dose. If a solute is present which is sensitive to H_2O_2 part of the radiation effect might be due to H_2O_2 formed during irradiation. On extrapolating the curves published by these authors to the two doses of α radiation used in the present experiment, one arrives at H_2O_2 concentrations of 7.35×10^{-3} and $3.68 \times 10^{-3}\text{M}$, which are the average concentrations of H_2O_2 built up during the experiment in the nuclear reactor. These concentrations are maximum concentrations, since one can assume that the presence of a solute reacting with OH radicals and/or H atoms will reduce the formation of H_2O_2 .

RESULTS

The results of the α radiation are summarized in Table 1. The ionic yields of the heavy particle radiation are 0.56 and 0.17 for the 200,000 and the 10,000 $\mu\text{g/ml}$ solutions respectively. Comparing these with the corresponding yields for X radiation (Dale, Davies & Gilbert, 1949) the relative efficiencies for deamination by α radiation and X radiation were 19.5% for the 20% solution, and 15.2% for 1% solution.

Table 2 shows the ammonia yield due to hydrogen peroxide. The hydrogen peroxide effect, therefore, is at most 12.5% of the α ray effect for the 200,000 $\mu\text{g/ml}$ glycine solution and 7.5% for the 10,000 $\mu\text{g/ml}$ solution.

Table 1 *Deamination of glycine by α particles*

Conway unit	Block 1			Block 2			Block 3		
	A ₁	B ₁	C ₁	A ₂	B ₂	C ₂	α	β	γ
Glycine concn ($\mu\text{g/ml.}$)		200,000			200,000			10,000	
KBO ₃ concn (M)	2 25	0	1 13	2 25	0	1 13	2 25	0	1 13
Total K concn (M)	2 25	2 96	2 60	2 25	2 96	2 60	2 25	2 96	2 60
NH ₃ yield ($\mu\text{g/ml.}$)	800	73	539	968	65	462	312	26	180
Blank ($\mu\text{g/ml.}$)	12	3	12	12	3	12	1	1	2
Net NH ₃ yield ($\mu\text{g/ml.}$)	788	70	527	956	62	450	311	25	178
NH ₃ yield due to α rays ($\mu\text{g/ml.}$)	718	—	457	894	—	388	286	—	153
Mol NH ₃ /ml $\times 10^{-18}$	2 57	—	1 64	3 20	—	1 39	1 02	—	0 55
Potassium activity* $\times 10^{-5}$	1 36	1 64	1 31	1 20	1 35	1 17	1 48	1 74	1 48
Neutron flux $\times 10^{-10}$	1 02	1 24	0 99	0 90	1 02	0 89	1 12	1 31	1 12
Ion pairs/ml $\times 10^{-19}$	5 64	—	2 76	4 99	—	2 47	6 20	—	3 10
Ionic yield for α radiation	0 46	—	0 56	0 64	—	0 56	0 16 ₅	—	0 17 ₇
Mean ionic yield			0 56					0 17 ₇	
Ionic yield for X radiation†			2 89					1 13	
Ratio (' α yield' as % of 'X yield')			19 5%					15 2%	

* Potassium activity is the counts/min/ml /'molar' K, 58 hr after the end of the irradiation

† Dale, Davies & Gilbert (1949)

Table 2 *Deamination of glycine by hydrogen peroxide at 31° during 21 hr*

Glycine concn ($\mu\text{g/ml.}$)	200,000		10,000	
H ₂ O ₂ concn (mm)	7 35	3 68	7 35	3 68
NH ₃ yield ($\mu\text{g/ml.}$)	104	67	25	11
Blank ($\mu\text{g/ml.}$)	12	12	1	1
NH ₃ due to H ₂ O ₂ ($\mu\text{g/ml.}$)	92	55	24	10

DISCUSSION

In Table 3 are set out the ammonia yields from the boron free solutions, which will be due to radiation by electrons and protons, reduced to a standard neutron flux of $10^{10}/\text{cm}^2/\text{sec}$. On the assumption that the ionic yield for these radiations is the same as for X radiation, the total dose and the dose rate in r/min has been calculated. This estimation, however, only gives a lower limit to the radiation dose, because the proton part, having a higher ion density, is probably not so efficient in deamination as the electron part, but there is reasonable agreement between the values calculated from solutions of different concentrations.

Table 3 *Effect of background radiation*

Glycine concn ($\mu\text{g/ml.}$)	Block 1	Block 2	Block 3
	200,000	200,000	10,000
NH ₃ yield/ml. for neutron flux of $10^{10}/\text{cm}^2/\text{sec.}$	57	61	19
Mol NH ₃ /ml $\times 10^{-18}$	0 70	0 75	0 61
Total dose $\times 10^{-5}$ (r)	4 35	4 60	3 78
Dose rate in flux 10^{10} r/min	347	372	302

In any comparison between radiation effects produced by the boron disintegration and those by α

particles from radon or polonium account must be taken of the different densities of the ions along the tracks of the particles. The boron disintegration produces two ionizing particles, an α particle with range in standard air of 0.77 cm or 7.7μ in water and a Li particle of range 0.48 cm in air or 4.8μ in water (Gilbert, 1948). The maximum ion density of the latter is only just greater than that of the α particle (Bower, Bretscher & Gilbert, 1938) because its range is shorter than the Bragg maximum in the ionization curve for lithium particles. The mean linear density of ionization along the disintegration track is 5900 ion pairs/ μ of track in water, the mean density of the lithium particle being about 13% less than in the α track. For comparison the corresponding figure for the mean of the three α particles emitted by radon and its short lived daughter products is 3500 ion pairs/ μ and for the electrons produced by a 200 kV X ray tube 80 ion pairs/ μ (Gray, 1947).

The relative yield for α particles compared with X radiation (15–19%) for deamination of glycine is considerably higher than the values (3–10%) for a range of concentrations for the inactivation of carboxypeptidase by α particles from radon and its short lived products (Dale, Gray & Meredith, 1949). The increased efficiency in the present experiment is not likely to be due to the fact that the linear ion density was greater than in the radon experiment, for this should rather result in a decreased efficiency. Both experiments show an increase of the ionic yields for α radiation with concentration of solute, though not proportional to concentration. The high value of 0.56 obtained for the highest concentration of glycine is of particular interest showing that the whole effect cannot be attributed to the δ rays but

that the main column of the α -track must play an important part

Bonet Maury & Lefort (1948) have shown that hydrogen peroxide is formed in pure water by α -radiation, and therefore part of the α radiation effect could be due to the presence of hydrogen peroxide if the solute is sensitive to it. The presence of a solute however may reduce the formation of hydrogen peroxide by reacting with the primary products of radiations (OH radicals and H atoms). Carboxypeptidase was shown to be insensitive to high concentrations of hydrogen peroxide (Dale, Gray & Meredith, 1949) and though the hydrogen peroxide can deaminate glycine, the effect is not more than 12% of the total radiation effect as shown by the present control experiments.

Since neither the quality of the radiation nor the effect of hydrogen peroxide can wholly account for the observed differences, one should assume that these are linked in some way with the fact that the X radiation effects on amino acids were very different from those on macromolecules, as was fully discussed in an earlier paper (Dale, Davies & Gilbert, 1949). There a chain reaction or the action of radicals by excitation was invoked for explaining the kinetics of

the deamination of amino acids by X-rays, and it was also pointed out that these theoretical considerations are not yet fully in agreement with current concepts of the indirect action of radiation.

SUMMARY

1 The deamination of glycine by the densely ionizing particles from the disintegration of boron by slow neutrons in a nuclear reactor has been studied. An account of the necessary technique is given and the dosimetry fully discussed.

2 The ionic yields for the liberation of ammonia were 0.17 and 0.56 for 1 and 20% solutions of glycine, respectively, being 15 and 19% of that due to X-radiation. The higher efficiency of deamination by this radiation compared with the inactivation of an enzyme by α rays from radon and its products is discussed.

We wish to thank the Director, Atomic Energy Research Establishment, Harwell, for permission to make the irradiation in the nuclear reactor and the staff of the Isotope Division for information concerning the neutron flux, for a standardized solution of ^{42}K and for the assistance and facilities given during the irradiation.

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Observations on Antisera Against Complexes of Di-2-chloroethyl sulphide and Protein

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(Received 31 January 1949)

Human beings and animals, after receiving repeated skin exposures to di-2-chloroethyl sulphide (mustard gas, 'H'), often develop an increased sensitivity to this substance, and it was felt that the factors concerned might be investigated by methods developed in the study of immunity. Berenblum & Wormall (1939) previously published an account of an investigation undertaken with this end in view, and the

present communication provides additional information about the system first described by these authors. It was also hoped that by studying different derivatives of diethyl sulphide as inhibitors in a specifically precipitating immunity system, some evidence might be obtained for the nature of the linkage formed when H reacts with proteins.

There have appeared in recent years a number of reports describing the action of *H* on proteins, e.g. Banks, Bournsell, Francis, Hopwood & Wormal (1946), Bournsell, Francis & Wormal (1946b), Herriott, Anson & Northrop (1946), and Ogston (1948). The serological investigation which forms the subject of the present communication has disclosed another factor in such reactions, viz the participation of inorganic phosphate.

EXPERIMENTAL

Preparation of compounds

Di 2 (2' amino 2' carboxyethylthioethyl) sulphide Prepared by interaction of *H* with cysteine in the manner described by Hellerman (1942), this had *m p* 245°*.

1 4-Thiazan-4-acetic acid and 2 hydroxy 2'-glycino-diethyl sulphide Ethyl aminoacetate and *H* were brought into reaction in absolute ethanol in the presence of anhydrous sodium acetate, and the crude oily product was isolated following the method of Cashmore & McCombie (1923). Two fractions were obtained from this product, one with *b p* 91–92°/1 mm, the other with *b p* 100–140°/1 mm. Each of these fractions was dissolved in dry ether, dry HCl gas was bubbled through the solution and the crystalline hydrochloride which separated in each case was collected on the filter with suction and recrystallized from an ethanol ether mixture (2/5).

From 10 g of the oil (*b p* 91–92°/1 mm) 10 g of recrystallized hydrochloride with *m p* 185–187° were obtained (Found C, 42.5, H, 7.1, N, 6.2, S, 14.4, Cl, 15.7%. The hydrochloride of ethyl 1 4-thiazan-4-acetate, $C_8H_{10}O_4NS$, requires C, 42.6, H, 7.2, N, 6.2, S, 14.2, Cl, 15.8%). This ethyl ester hydrochloride (5 g) and *N* NaOH (100 ml) were refluxed for 4 hr, after which the mixture was cooled, *N* H_2SO_4 (78 ml.) was then added and the solution distilled to dryness *in vacuo*. The residue was extracted three times with boiling ethanol, the extracts filtered and reduced to dryness, and the residue crystallized from ethanol. Yield, 3.0 g. The resulting needle shaped crystals showed a peculiar behaviour in melting point determinations. In a capillary tube they melted at 130°, above this temperature some samples decomposed with much evolution of gas, while other samples recrystallized and melted at 175°. On the heated stage of a Fisher-Johns micro melting point apparatus they had *m p* 175–176° with no preliminary melting at 130°. This transient melting at 130° was probably due to loss of water of crystallization, since analyses showed that the crystals contained water. A sample of the crystals was sublimed at 160°/0.15 mm, the sublimate melted and turned red at 177–179° without any preliminary melting at 130° (Found for the sublimate C, 44.5, H, 7.4, N, 8.5, S, 20.2. Calc for $C_6H_{10}O_4NS$, C, 44.7, H, 6.9, N, 8.7, S, 19.9%). There seems little doubt that this material is identical with the 1 4-thiazan-4-acetic acid described by Bournsell, Francis & Wormal (1946a).

From 7 g of the oil (*b p* 100–140°/1 mm.) 2 g of recrystallized hydrochloride with *m p* 120–122° were obtained (Found C, 42.0, H, 6.9, N, 5.1, S, 11.4, Cl, 12.1. Calc for the ethyl ester hydrochloride of 2 acetoxy 2

glycino diethyl sulphide, $C_{10}H_{20}O_4NCIS$, C, 42.1, H, 7.05, N, 4.9, S, 11.4, Cl, 12.4%). This ethyl ester hydrochloride (2 g) and *N* NaOH (30 ml) were refluxed for 2 hr, after which the mixture was cooled, *N* H_2SO_4 (20 ml) was added and the solution was distilled to dryness *in vacuo*. The residue was extracted three times with boiling ethanol, the extracts filtered and reduced to dryness, and the residue crystallized by dissolving it in 10 ml of hot ethanol, adding 2 ml. of acetone and chilling. The crystals separated as rosettes of needles melting at 129–131°. The material was very soluble in water, ethanol and methanol, but relatively insoluble in acetone, ether and benzene (Found C, 40.3, H, 7.4, N, 7.8, S, 18.0. Calc for $C_6H_{12}O_4NS$, C, 40.2, H, 7.3, N, 7.8, S, 17.9%).

The analyses of this material and of the ester hydrochloride from which it was derived provide good, though not conclusive, evidence that it is 2 hydroxy 2' glycino-diethyl sulphide, and it is quite conceivable that the acetate of the ethyl ester of this compound could have arisen as a result of the reaction of 1 mol. of *H* simultaneously with 1 mol. of sodium acetate and 1 mol. of ethyl aminoacetate.

In the course of several preparations of the sort just described many attempts were made to obtain the di 2 glycinoethyl sulphide described by Cashmore & McCombie (1923), but without success.

H glycine† Glycine (10 g), water (200 ml.) and *H* (30 ml) were placed in a 500 ml. beaker and stirred continuously at 37°, 2*N* NaOH being added at a rate sufficient to keep the reaction alkaline to phenolphthalein. After 8 hr the consumption of alkali had ceased when 260 ml of 2*N* NaOH had been added. The reaction mixture was then extracted three times with chloroform and the chloroform washings discarded. The aqueous solution was concentrated *in vacuo* on the steam bath to about 100 ml when a large amount of NaCl had separated, the sludge in the distilling flask was filtered with suction and the solid on the filter washed with a little cold water. The pH of the filtrate was adjusted to about 2 by the addition of conc HCl. As the acidification proceeded a copious, gummy, slightly yellow precipitate formed and rapidly settled to a gummy mass at the bottom of the flask. After the mixture had been chilled for 2 hr at 5° the supernatant liquid was decanted and discarded and the gummy precipitate washed twice with cold water. The remaining gum was then dissolved in 100 ml water by adding 2*N* NaOH until the pH of the solution was 6.5. The precipitation with acid, washing and redissolving of the product was carried out twice more the resulting gum was dissolved in water at pH 6.5 and the solution diluted to 50 ml. This solution had 5.3 mg N/ml., yield, 14% (based on *N*).

A solution which had been made from a similarly prepared and purified reaction product gave total N (Kjeldahl) 0.93 mg./ml., amino N (Van Slyke), 0.00 mg./ml., S, 1.46 mg./ml. When 5 ml of this solution was mixed with 0.1 ml. of a 10% solution of any one of cobaltous acetate, cadmium chloride, nickel nitrate or cuprous sulphate a copious precipitate formed. The chemical properties of this *H glycine* received considerable study which will be the

† The '*H glycine*' referred to here is not to be confused with the '*H glycine*' used by Bournsell *et al* (1946a) to mean 1 4-thiazan-4-acetic acid, but refers to the unidentified product of the reaction of *H* and glycine in aqueous solution.

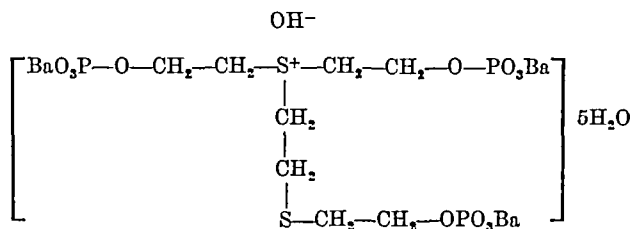
* All *m p* are uncorrected

subject of a communication by Drs D E Douglas and R D H Heard

'Thiodiglycol phosphate.' $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (6.7 g) was dissolved in water (100 ml) and the solution was maintained at 37° , 4 g of *H* (m.p. 14°) were then added and the reaction mixture stirred continually. The reaction was kept just alkaline to phenolphthalein by the addition of 2N NaOH. After 2 hr, when the reaction was over, 10 ml of 2N NaOH had been used and an analysis for inorganic

On electrometric titration of the barium salt of 'thiodiglycol phosphate' and sodium phenyl phosphate it was found that the former bound more alkali between pH 9 and 12.2 than did the latter. The extra alkali bound was approximately one equivalent for three atoms of phosphorus.

In view of the above findings and the fact that *H* in aqueous solution readily gives rise to sulphonium salts (Herriott, 1946), the structure proposed for the barium salt of 'thiodiglycol phosphate' is



phosphate showed that 80% of that originally present had been esterified. The reaction mixture was distilled to dryness *in vacuo* and the residue was washed with absolute ethanol, the washings being discarded. The residue was then dissolved in 5 ml of water, 50 ml of absolute ethanol were added and the mixture was chilled at 5° for 3 hr when the clear supernatant liquid was decanted and discarded. The solution and precipitation were carried out twice more after which the residue was dissolved in water and diluted to 50 ml. This solution gave P (total), 12.5 mg/ml, P (inorganic), 3.6 mg/ml, S, 8.2 mg/ml.

The above solution was made alkaline to phenolphthalein by the dropwise addition of a saturated $\text{Ba}(\text{OH})_2$ solution, the precipitate which formed was centrifuged and discarded. To the supernatant fluid $\text{Ba}(\text{OH})_2$ dissolved in hot water was added in the proportions of one atom of Ba for each atom of P. The precipitate which resulted was centrifuged and washed three times with small volumes of distilled water. To the combined supernatant and washings, which were perfectly clear and colourless, an equal volume of ethanol was added and the resulting voluminous precipitate centrifuged, washed twice with 50% and twice with 95% ethanol, and dried *in vacuo* over CaCl_2 . (Found C, 10.0, H, 2.8, S, 6.3, P, 9.5, Ba, 41.2. $\text{C}_8\text{H}_{16}\text{O}_{13}\text{S}_2\text{P}_2\text{Ba}_2 \cdot 5\text{H}_2\text{O}$ requires C, 9.8, H, 2.7, S, 6.6, P, 9.5, Ba, 42.1%.)

It can be seen that the ratio P/S in this material is 3:2 and at one time it was suspected that this might be due to a mixture of thiodiglycol mono and di phosphates. In order to test this possibility a brucine salt was prepared and analysed for P and S. Aqueous solutions of the above Ba salt (0.5 g) and brucine sulphate (1.5 g) were mixed, BaSO_4 was removed by centrifuging and the resulting solution was evaporated *in vacuo*. Crystallization of the residue from water yielded 0.663 g of fine white needles. Even after recrystallization this brucine salt had no definite m.p. but softened, melted and decomposed gradually from 110 – 185° (Found S, 2.32, P, 3.35%). These analytical results indicate that the Ba salt was a single compound.

On drying the Ba salt in the pistol *in vacuo* over P_2O_5 it lost weight, but quantitative water determinations were impossible. The weight loss increased steadily as the temperature in the pistol was raised from 100 to 190° , until at the latter temperature the material turned brown and was obviously decomposing.

If the substance contains a sulphonium salt grouping it should be in the form of the sulphonium hydroxide when it separates from the alkaline baryta solution. The slightly high carbon content found on analysis might be due to a partial neutralization of the sulphonium hydroxide with CO_2 . It seems impossible to reconcile the analytical results with any other reasonable structure, but unfortunately the proposed constitution does not readily lend itself to proof by degradation or synthesis.

For use in specific inhibition tests the Ba salt was dissolved in water and Ba removed by the careful addition of 1% Na_2SO_4 solution to slight excess. The resulting solution, after removal of BaSO_4 and adjustment of volume, was used as an inhibitor.

Reaction of H with serum proteins Preparations were made from the serum proteins of the horse, rabbit, guinea pig, pig and man.

(a) *H* horse serum globulin (phosphate, pH 8–9) Horse serum globulin solution (50 ml, containing 0.46 g N) was mixed with 0.5N phosphate buffer (25 ml, pH 8) and warmed to 37° . *H* (3 ml) was added and the mixture stirred constantly and maintained at 37° . 2N NaOH solution was added at such a rate as to keep the mixture just alkaline to phenolphthalein. After 8 hr the reaction was finished and 10 ml of 2N NaOH solution had been used. The resulting solution was placed in a cellophane sac and dialysed against running tap water for 48 hr. The contents of the sac were clarified by filtering, and analyses for N and S were carried out on the solution. (At first, samples of the solution of antigen were precipitated with acetone and the precipitate, after having been washed, and dried *in vacuo*, was analysed for S and N. Since it was later found that the same values for S/N were obtained when the analyses were carried out on samples of solution, the acetone precipitation procedure was discontinued.) In each case, analyses for S and N were carried out on the original protein before treatment with *H*. Analyses for the above coupling were: original horse serum globulin solution S/N, 0.09, coupled horse serum globulin solution S/N, 0.34, Δ (S/N), 0.25. The final solution was stored at 5° with a few crystals of thymol as a preservative.

(b) *H* horse serum albumin (phosphate, pH 8–9) Horse serum albumin solution (75 ml, 8.5 mg N/ml), 0.5M phosphate buffer (33 ml, pH 8.0) and *H* (4 ml) were

stirred at 37° 2N NaOH (27.5 ml) was added at a rate sufficient to keep the reaction just alkaline to phenolphthalein. After 4 hr the reaction was finished and the resulting mixture was dialysed in a cellophane sac against running tap water for 4 days. The contents of the sac were then filtered and the filtrate electro dialysed against running distilled water until no current flowed through the cell. Early in the dialysis, a gelatinous precipitate began to form and this increased in amount throughout the operation. The dialysed mixture was centrifuged and the supernatant fluid (215 ml) had a pH of 4.5 and contained 0.07 mg N/ml and 0.000 mg total P/ml, it was discarded. The gelatinous precipitate was washed once with distilled water, centrifuged and the washing discarded. The precipitate was treated with 150 ml water and enough N NaOH to bring the pH to 9.0. The mixture was stored at 5° overnight when the pH had become 7.0. A trace of undissolved gel was removed by centrifuging and discarded. The supernatant solution (100 ml) had N, 3.43 mg/ml, P (total), 0.25 mg/ml, P (inorganic), 0.011 mg/ml, S, 1.29 mg/ml, S/N, 0.38. The original horse serum albumin solution had S/N, 0.12.

(c) *H* horse serum albumin (phosphate, pH 7.5) A preparation of *H* serum albumin solution was made by reacting 63 ml. of 5.3% horse serum albumin solution, 28 ml. of 0.5M phosphate and 3.4 ml. of *H* at 37° and pH 7.5. The pH, measured by a glass electrode inserted in the reaction vessel, was kept constant at 7.5 by the addition of NaOH. After dialysis and electro dialysis, carried out as in the above preparation, the resulting solution had $\Delta(S/N)$, 0.11 and P/N , 0.070. In this preparation 5% of the phosphate was present as inorganic phosphate.

(d) *H* guinea pig serum (no buffer, pH 8-9) Dialysed fresh guinea pig serum (22 ml), water (20 ml.) and *H* (0.5 ml) were stirred 8 hr at 37°. The solution was kept alkaline to phenolphthalein by the addition of 2N NaOH solution. The reaction mixture was dialysed against running tap water for 48 hr and filtered, the product had $\Delta(S/N)$, 0.29.

(e) *Controls* Table 1 shows the results obtained in control experiments. In each case the reactants were contained in 75 ml water and stirred at 37° for 8 hr at pH 8-9. The resulting solution was dialysed against running tap water for 70 hr and then electro dialysed to zero current against distilled water.

Table 1 *Fixation of sulphur and phosphate by horse serum globulin*

Exp	Reactants	Analyses of product	
		S/N	P/N
LXIII	Na ₂ HPO ₄ (0.0125 g mol), horse serum globulin (2.8 g)	0.12	0.005
LI	'Thiodiglycol phosphate' (0.0125 g mol. P) horse serum globulin (2.8 g)	0.12	0.041
LII	'Thiodiglycol phosphate' (0.0125 g mol. P), horse serum globulin (2.8 g) <i>H</i> (1 ml.)	0.24	0.062

The product of Exp. LII only was precipitated by the appropriate antiserum (Table 2)

Experiments with phosphatase Two different phosphatase preparations were used, one was an aqueous extract of rat kidney, the other a solution of an active powder prepared from dog faeces (Armstrong, 1935). For use 10 mg of this intestinal phosphatase preparation was dissolved in 20 ml water. The rat-kidney extract was prepared by grinding fresh, decapsulated rat kidneys in a mortar with sand and five times their weight of water, the resultant brew was stored at 5° overnight and centrifuged. The supernatant liquid was diluted twenty times with water for use.

At pH 9.5 (veronal buffer of King & Delory, 1940) and 37° with 1 mg of phosphate as 'thiodiglycol phosphate' and 5 ml of enzyme solution in 15 ml of digest, hydrolysis with the kidney phosphatase was 63% complete at 5 hr and 100% complete at 22 hr, hydrolysis with the intestinal phosphatase was 95% complete in 6 hr and 99% complete in 22 hr.

Under the same conditions, with the *H* horse serum albumin (phosphate) prepared at pH 8-9, as described in the previous section, the intestinal phosphatase gave 13% hydrolysis of phosphate at 22 hr and the kidney phosphatase gave 7% hydrolysis at 22 hr. The *H* horse serum albumin (phosphate) prepared at pH 7.5 on treatment with the intestinal phosphatase under the same conditions gave 28% hydrolysis of phosphate in 22 hr. Both these solutions, after treatment with intestinal phosphatase, were electro dialysed to zero current and analysed. The analyses showed that the S/N values had not been altered by the phosphatase treatment, but that the P/N values had been reduced by 20%.

The pH 8-9 preparation before and after phosphatase treatment was precipitated by antiserum and the precipitations were inhibited by 'thiodiglycol phosphate' (Tables 2 and 4).

Immunization experiments

Various courses of immunization were carried out by injecting antigens prepared by method (a) into young adult rabbits. In general, it was found that an intravenous course gave a fairly good antiserum, if this were followed by an intraperitoneal course the antiserum obtained was much weaker, but if the intraperitoneal course were followed by a second intravenous course the antiserum was once more strongly precipitating.

Antigens prepared by method (a) from a number of different serum proteins fell into the following order of effectiveness: pig serum globulin, pig serum, horse serum globulin, horse serum, rabbit serum, human serum globulin, human serum.

The above general conclusions were arrived at from experiments on 100 rabbits. As a result of these experiments, we settled on the following procedure which was expedient and almost invariably gave strongly precipitating antisera. Rabbits were given a course of four intravenous injections, each containing about 8 mg of antigen nitrogen, at 4-day intervals, and were bled out 3-10 days after the last injection. The best results were obtained when coupled pig serum globulin prepared by method (a) was the antigen.

The immunological experiments carried out with antigens prepared by method (c) were too few to justify any conclusions. All attempts to produce satisfactory antisera for antigens prepared by method (d) failed, a large number of such attempted immunizations were made by various procedures including mixing the antigen with adjuvants according to the method of Freund & McDermott (1942).

Precipitin tests These tests were carried out by mixing 0.5 ml of antigen solution in increasing dilutions (1/5 to 1/2560) with 0.5 ml of antiserum in constant dilution (1/5), the diluent was 0.85% NaCl. All readings were made at room temperature. After the test was set up frequent readings were made to determine which tube first showed definite flocculation, this established the optimal antigen/antibody ratio. The test was then continued at room temperature overnight after which time a final reading of the precipitation was made.

Table 2 summarizes the results of precipitin tests of the sera of rabbits immunized with *H* proteins prepared by method (a). These data are a representative selection from a large number of such tests.

Inhibition tests A sample of strongly precipitating

rabbit serum was produced by injecting *H* coupled horse serum globulin prepared by method (a), and this antiserum was titrated with decreasing concentrations of coupled guinea pig serum prepared by method (a). The optimum concentration of antigen for reaction with a 1/5 dilution of antiserum was 0.025% protein. Inhibition tests were carried out on this optimal system as follows: 0.25 ml of solutions with decreasing concentrations of the various inhibitors were incubated for 1 hr at 37° with 0.25 ml of a 1/2.5 dilution of antiserum, the resulting mixtures were then tested for precipitation with 0.5 ml of coupled guinea pig serum protein solution containing 0.025% N. The inhibitors tested in this manner were neutral solutions of thiodiglycol, thiodiglycol and sodium phosphate, 2-hydroxy-2'-glycino-diethyl sulphide, 1.4-thiazan-4-acetic acid, 'thiodiglycol phosphate' and *H* glycine, the di-2 (2'-amino-2'-carboxyethylthioethyl) sulphide was dissolved at pH 9 since it would not dissolve at a lower pH. The only solution which produced any inhibition was that of 'thiodiglycol phosphate', the results of a typical inhibition test with this material are to be seen in Table 3.

Table 2 *Precipitin reactions of rabbit antisera*

(The bracketed letters (a), (b), (d), (e) refer to the subsection in which the preparation of the complex is described. *h s a* and *h s g* = horse serum albumin and globulin, respectively, *r s* = rabbit serum, *g p s* = guinea pig serum, *p s g* = pig serum globulin.)

Rabbit no	Immunizing antigen	Antiserum (1/5) titrated with	Optimal antigen conc (% protein)	Time of appearance of ppt	Amount of ppt
1321	<i>H h s g</i> (a)	<i>H h s g</i> (a) (phosphate, pH 9)	0.2	2 min	++++
1321	<i>H h s g</i> (a)	Normal horse serum	0.1	4 hr	+
1321	<i>H h g s</i> (a)	<i>H r s</i> (a) (phosphate, pH 9)	0.2	Immediate	++++
1321	<i>H h s g</i> (a)	Normal rabbit serum		No ppt	
A24	<i>H h s g</i> (a)	<i>H g p s</i> (a) (phosphate, pH 9)	0.002	1 min	++++
A24	<i>H h s g</i> (a)	<i>H g p s</i> (d) (no buffer, pH 9)	0.002-0.03	2 hr	++
1353	<i>H r s</i> (a)	<i>H h s g</i> (a) (phosphate, pH 9)	0.03	15 min	+++
J74	<i>H p s g</i> (a)	<i>H h s a</i> (a) (phosphate, pH 9)	0.2	Immediate	++++
J74	<i>H p s g</i> (a)	<i>H h s a</i> (d) (no buffer, pH 9)		No ppt	Opalescence in all tubes
A72	<i>H p s g</i> (a)	<i>H h s a</i> (b) (phosphate, pH 9, electro-dialyzed)	0.1	1 min	++++
A72	<i>H p s g</i> (a)	<i>H h s a</i> (b) (same as above but after phosphatase treatment)	0.03	1 min	++++
A72	<i>H p s g</i> (a)	<i>H h s g</i> LII (e) ('thiodiglycol phosphate', pH 9)	0.5	1 min.	++++

Table 3 *Inhibition test using 'thiodiglycol phosphate'*

(Antiserum to *H* horse serum globulin (phosphate, pH 9). Test antigen *H* guinea pig serum (phosphate, pH 9). Each tube contained 0.25 ml of antiserum (1/2.5), 0.25 ml of inhibitor solution and 0.5 ml of antigen (0.025%). Degrees of precipitation: op, opalescence without particulation, tr, trace of precipitate, ±, +, ++, etc., increasing degrees of precipitation.)

Tube no	1	2	3	4	5	6
Conc of inhibitor (mg P/ml.)	2.0	1.0	0.5	0.25	0.125	
Saline (ml.)						0.25
Precipitation observed at 30 min.				op	op	++++
2.5 hr			op	op	op	++++
6 hr			op	tr	±	++++
20 hr		tr	+	++	++	++++

Table 4 *Effect of 'thiodiglycol phosphate' on precipitin reactions*

(Bracketed letters refer to methods of preparation)

Rabbit antiserum to	Test antigen	Inhibition
<i>H</i> horse serum globulin (a) (phosphate, pH 9)	<i>H</i> guinea pig serum (a) (phosphate, pH 9)	+
<i>H</i> horse serum globulin (a) (phosphate, pH 9)	<i>H</i> guinea pig serum (d) (no buffer, pH 9)	-
<i>H</i> horse serum globulin (a) (phosphate, pH 9)	<i>H</i> rabbit serum (a) (phosphate, pH 9)	+
<i>H</i> horse serum globulin (a) (phosphate, pH 9)	<i>H</i> horse serum albumin (b) (phosphate, pH 9, electro-dialysed)	+
<i>H</i> pig serum globulin (a)	<i>H</i> horse serum globulin LII (e) ('thiodiglycol phosphate', pH 9)	+
<i>H</i> pig serum globulin (a)	<i>H</i> horse serum albumin (a) treated with phosphatase	+
Normal horse serum	Horse serum globulin	-
Hen egg albumin	Hen egg albumin	-

The effects of 'thiodiglycol phosphate' in a number of different precipitating systems are recorded in Table 4

DISCUSSION

The qualitative effect of the presence of sodium phosphate on the reaction of *H* with protein was first suggested by the results of the inhibition tests, although a quantitative effect had been noted previously. It had been found in our experiments that four times as much *H* could be brought into reaction with a protein in the presence of sodium phosphate to yield a soluble product as when the reaction was carried out under similar conditions but with no buffer present, thus was exclusive of the amount of *H* reacting with the phosphate itself. Subsequent chemical analyses then established the fact that when *H* reacted with proteins in the presence of sodium phosphate at pH 7.5 or 9, complexes were formed which contained residues of diethyl sulphide, phosphate and protein.

The presence of ester phosphate in the *H* protein phosphate complexes might possibly have been due to the adsorption by the protein of some 'thiodiglycol phosphate', although this seems unlikely in view of the serological findings and the vigorous dialysis treatment which some of the complexes received before analysis. In order to obtain more information on this point, however, one complex prepared at pH 8-9 and one at pH 7.5 were treated with phosphatase. Although the two phosphatase preparations both hydrolysed the 'thiodiglycol phosphate' rapidly and completely they had only a small effect on the antigens under similar conditions.

The *H* phosphate protein complexes are highly antigenic, and the groups introduced are powerfully determinant in the immunological sense, since such complexes formed from rabbit serum are antigenic for rabbits. There is no direct evidence for the configuration of the determinant group introduced, but one possibility is that it is the same as the 'thiodiglycol phosphate' but with protein substituted for one of the phosphate groups. In this connexion it is

important to note that some phosphate, but no sulphur, is fixed by the protein in the presence of 'thiodiglycol phosphate' and that the product does not react specifically with antiserum to *H* protein (phosphate). This small amount of phosphate fixed in Exp. LI (Table 1) may be related to the fraction of fixed phosphate liberated by phosphatase from the complexes, in the latter case the phosphate content of the complex was reduced without a reduction in the sulphur content.

It is most unlikely that the *H* phosphate protein complexes referred to above are the only products of reaction of *H* with protein in our experiments. A number of diethyl sulphide groups could have been introduced into the protein without the intervention of phosphate, and these could be similar to those introduced when *H* reacts with protein with no buffer present. Such a conclusion is indicated by the results obtained with antiserum A24 (Table 2). Under the conditions of our experiments, such groups showed very little capacity for producing a specific antibody response, and this lack of antigenic activity is similar to that found by Bournsnel *et al* (1946b) for their *H* serum protein complexes. These findings make it very difficult to interpret the part played by *H* protein derivatives in the phenomenon of hypersensitivity to mustard gas.

A number of experiments were carried out to explore the possibility that the reaction of mustard gas simultaneously with inorganic phosphate and protein was of some significance in the mechanism of action of mustard gas on living cells, but all the findings were negative. The results of our work probably have a bearing on some other biochemical investigations of the mechanism of mustard gas action such as the effects of mustard gas on enzymes and other proteins *in vitro*. In the case of phosphate, at least, the buffer could play a prominent part.

SUMMARY

1 Two pure substances, 1.4-thiazan-4-acetic acid and (probably) 2-hydroxy-2'-glycino-diethyl sulphide, have been isolated from the product of re-

action of mustard gas (*H*) with ethyl aminoacetate in anhydrous ethanol in the presence of sodium acetate

2 The reaction of *H* with glycine in aqueous solution at pH 9 yields a water soluble product with unusual properties

3 *H* reacts extensively with sodium phosphate in aqueous solution, analyses of the reaction product indicate that it is a sulphonium salt derived from 2 mol of *H* and containing three phosphate ester groups

4 It has been found that when *H* reacts with serum proteins in the presence of sodium phosphate at pH 8-9, complexes are formed which contain, in addition to an increased concentration of sulphur, appreciable amounts of esterified phosphate which cannot be removed by electrodialysis

5 When these complexes are injected intravenously into rabbits they give rise to strongly precipitating antisera

6 The precipitin reactions between these coupled proteins and the appropriate antisera are inhibited by the reaction product of *H* and sodium phosphate, but not by a number of other derivatives of diethyl sulphide

7 The complexes formed when *H* reacts with serum proteins at pH 8-9 with no buffer present have been found to be ineffective in producing serum precipitins in rabbits

Microanalyses of the reaction products of mustard gas and ethyl aminoacetate were carried out by Mrs Barker through the kindness of Dr Leo Marion, Chemistry Division, National Research Council, Ottawa. Carbon and hydrogen contents of the 'thiodiglycol phosphate' were measured by Dr A. Elek, Los Angeles, California.

Acknowledgement is made to Mr J. M. Chaput for his skilful technical assistance. Thanks are due to Prof. E. G. D. Murray for his continued interest and encouragement, Prof. A. M. Wynne for helpful advice, and Dr A. R. Armstrong for a gift of intestinal phosphatase.

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Investigations into Cholinesterase Levels in Serum and Cerebrospinal Fluid of Psychotic Patients

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(Received 19 April 1949)

Claims have been made that the level of serum cholinesterase can be correlated with certain psychiatric conditions. This level is said to be raised in states of anxiety and depression, and lowered in catatonic stupor, schizophrenia and epileptic conditions (Jones & Tod, 1937; Jones & Stadie, 1939; Birkhauser, 1941a, b; Richter & Lee, 1942; Butt, Comfort, Dry & Osterberg, 1942; Schutz, 1944).

The data accumulated were quite often inconsistent and did not permit definite conclusions about the role of cholinesterase in disturbances of the nervous tissue. Mendel, Hawkins & Nishikawara (1948) assumed that this might be due to the fact that at the time when these earlier investigations were reported the existence of two enzyme systems

capable of hydrolysing acetylcholine was not known. Only later Mendel & Rudney (1943) and Mendel, Mundell & Rudney (1943) distinguished between the true cholinesterase and the pseudo cholinesterase and developed methods for measuring the activities of these enzyme systems. The authors claimed that only the true cholinesterase is essential for hydrolysis of acetylcholine *in vivo*.

The investigations reported in this paper were made because of certain therapeutic possibilities envisaged, which might occur when using different substances influencing cholinesterase activity. The following points were of particular interest: (1) Whether there existed any correlation between the values of true cholinesterase and the total brain size

and the number of functioning neurons (ii) Whether serum true cholinesterase varied in different mental disorders (iii) What changes of true cholinesterase levels took place in the serum after electrically induced convulsions (iv) What relationship existed between true and pseudo cholinesterase in the serum if changes of one of them occurred (v) Whether a relationship existed between true cholinesterase in the serum and in the cerebrospinal fluid (c s f) (True cholinesterase in the c s f has recently been reported (Reiss & Hemphill, 1948))

METHODS

The cholinesterase activity was determined manometrically, using Warburg manometers, and the results expressed in μ l. of CO_2 produced by 1 ml. serum or c s f in 30 min at 37.5° . The substrate concentrations used throughout the determination were 0.3M acetylcholine, 0.06M benzoyl choline, and 0.3M acetyl β methylcholine. The substrate (0.2 ml) was placed in the side compartment, and the c s f or serum in the main compartment, with 2% bicarbonate solution (0.2 ml) and distilled water sufficient to make the total fluid content up to 2.0 ml. For true cholinesterase determinations 1 ml. of serum was used, but for determinations with acetylcholine and benzoylcholine 0.1 or 0.05 ml serum was sufficient. Determinations with c s f required 1.6 ml., only clear sera or c s f were used. All estimations were made in duplicate, the average error being $\pm 1.4\%$. Blood was collected from the cubital vein. The usual technique for electroshock treatment was followed, a major epileptic fit being induced with standard Ediswan apparatus. Blood samples were taken before and 2, 15 and 60 min after the onset of the epileptic fit. The same procedure was repeated after several treatments.

RESULTS

Serum true cholinesterase determinations were made on fifty five male chronic psychotics (aged 59–85 years). The results were divided into three groups, showing those above (a), inside (b) and below (c) the normal range (Table 1). Of these patients 13 (24%) showed serum true cholinesterase values within the normal range, 31 (56%) gave high values and 11 (20%) low values. Twenty one middle aged schizophrenics and fifteen mental defectives gave normal values. It is remarkable that the cholinesterase value of the middle aged schizophrenics is relatively constant over a period of 3 weeks. One 9 year old mental defective, a hydrocephalic, gave high cholinesterase values (patient no. 6 in Table 3).

Some specimen values of the fifty five aged male psychotics for true and pseudo cholinesterase in serum and c s f are recorded in Table 2, while Table 3 shows true and pseudo cholinesterase values of mental defectives. Both tables show that no correlation could be found between the true cholinesterase and pseudo cholinesterase content of the

serum. There is also no correlation between the cholinesterase content of serum and c s f, the c s f cholinesterase content could be the same whether the serum level was 170 or 5. Tables 2 and 3 show also that low levels of serum or c s f cholinesterase were not related to small brains, as in defectives, or reduced cortical size, as in organic or senile dementia.

The serum cholinesterases of fifteen patients were investigated before and after electroconvulsion treatment. Representative results of five patients are recorded in Table 4. The effect of the electrically induced convulsions on the level of serum true cholinesterase was not the same for each patient. From 1 to 90 min after shock the true cholinesterase may rise, fall or remain constant. After repeated shocks, however, the serum true cholinesterase level of the patient (taken in the morning, under standard conditions, before treatment) showed a steady decrease. This decrease is greater than spontaneous changes seen in middle aged schizophrenics in the course of 3 weeks (cf. Table 1).

Only very slight change can be seen in the level of the pseudo cholinesterase after electroshock treatment. The two enzyme systems react quite independently. True cholinesterase could decrease, while pseudo cholinesterase showed a rising tendency, and vice versa.

DISCUSSION

No correlation between pseudo and true cholinesterase was found in any of the investigations described in this paper. This is in agreement with the recent findings of Mendel *et al.* (1948), who investigated these enzyme systems in the plasma of male rats after thyroidectomy and inanition. However, considering the quite different substrates hydrolysed by these two enzyme systems no close biological correlation was to be expected.

The changes produced by electrical convulsions in the true cholinesterase level may be the expression of a regulatory mechanism starting after increased acetylcholine production has taken place. One could assume that two factors are involved in this regulatory process: (1) immediately after an electrically induced fit a great part of the enzyme system is used by the nervous system for the hydrolysis of acetylcholine and disappears from the circulation, (2) new cholinesterase is produced as a compensation, and reaches the circulation. The chronological coordination between these two processes is decisive for the kind of change—decrease or increase—in the true serum cholinesterase after electroconvulsion treatment.

Some evidence was contributed by animal experiments. We found that after intravenous injection of 4–8 mg of acetylcholine into rabbits the true cholinesterase content of the serum 4 min after injection decreased in four experiments, was unchanged

Table 1 Serum true cholinesterase of some mental patients

(Cholinesterase activities are expressed in μ l CO₂ produced by 1 ml serum in 30 min)

Patients and controls	No investigated	Age		Serum true cholinesterase	
		Range	Average	Range	Mean \pm σ
Aged psychotics (a)*	31	50-76	66.9	62.8-233	116.2 \pm 43.5
	(b) 13	60-77	67.5	26.0-54.3	37.2 \pm 8.6
	(c) 11	65-84	75	0-22.4	6.9 \pm 8.1
Middle aged schizophrenics }	21	17-48	34.7	{22.4-48.6	31.6 \pm 6.47
Same patients 3 weeks later }				{22.4-45.4	34.7 \pm 6.69
Mental defectives	15	13-54	26.3	26.4-54.8	37.5 \pm 8.47
Normal controls	11	18-34	28.3	25.5-36.6	31.5 \pm 4.09

* See text

Table 2 Serum and cerebrospinal fluid cholinesterases of some male psychotics

(Cholinesterase activities are expressed in μ l CO₂ produced by 1 ml serum in 30 min ACh, acetylcholine, BCh, benzoylcholine, MCh, acetyl β methylcholine)

No	Patient	Age	Serum			Cerebrospinal fluid		
			ACh	BCh	MCh	ACh	BCh	MCh
4	H M.	62	1660	690	170	10.9	4.5	5.6
6	A.E.J	60	1671	402	167	14.7	5.4	9.0
9	F.W.B	71	3080	1000	110	11.0	2.1	3.85
21	E.E.A	72	3530	1457	87	15.4	0.83	5.6
26	E.N.P	76	1822	1458	63	11.5	2.52	5.54
29	A.J	60	2450	435	73	12.6	0	4.7
36	T.R	73	2080	875	37	14.7	1.7	5.7
48	E.A.	78	2240	1012	8.6	15.4	8.1	8.4
50	D.M	82	1767	662	0	21.0	0.94	9.2
52	W.G.H	65	2685	1155	8	9.2	0	5.1
53	T.W	71	1952	840	0	15.9	6.5	10.0

Table 3 Serum and cerebrospinal fluid cholinesterases of mental defectives

(Cholinesterase activities are expressed in μ l CO₂ produced by 1 ml. serum in 30 min ACh, acetylcholine, BCh, benzoylcholine, MCh, acetyl β methylcholine)

Patient no	Age	Cerebrospinal fluid			Serum			Diagnosis
		ACh	BCh	MCh	ACh	BCh	MCh	
1	29	11.3	3.3	6.8	2244	830	27.6	Imbecile
2	27	17.5	3.6	6.4	2656	1075	32.2	Imbecile (epileptic)
3	24	16.5	3.95	7.85	2531	1212	49.4	Imbecile
4	24	9.15	—	5.15	2599	1155	39.6	Feeble minded
5	28	14.3	4.2	6.8	2106	828	30.4	Imbecile
6	9	21.2	5.9	7.3	4606	2118	63.8	Hydrocephalic imbecile
7	13	9.35	2.58	—	2598	1110	36.3	Microcephalic
8	27	10.8	—	5.5	2522	835	29.5	Imbecile
9	20	9.55	4.9	4.7	2560	1080	39.7	Imbecile
10	19	8.6	2.3	3.5	2498	1058	37.9	Imbecile
11	54	11.1	0.7	6.3	2573	950	44.4	Microcephalic imbecile
12	17	9.8	3.4	4.8	2311	1388	30	Idiot
13	19	17.8	4.1	8.5	3773	1642	46.8	Imbecile
14	56	—	—	—	1961	842	26.4	Microcephalic imbecile
15	18	14.1	3.4	6.1	3123	1388	54.8	Imbecile
16	20	7.05	2.42	2.9	2873	1232	37.2	Mongol imbecile

in one, and increased in a sixth (work to be published elsewhere)

In most patients, after one electrically induced convulsion a lowered cholinesterase level could be seen on the next morning, and a further shock at this time would depress the cholinesterase level still

further We do not know to what extent this level can be depressed, nor do we know as yet whether many fits induced at short intervals would produce a rapid lowering of serum cholinesterase or, on the other hand, stimulate a compensatory mechanism, but we feel that this effect of electroshock is of

Table 4 Serum cholinesterases of patients before and after electroconvulsion treatment

(Cholinesterase activities are expressed in μ l, CO₂ produced by 1 ml serum in 30 min ACh, acetylcholine, BCh., benzoylcholine, MCh, acetyl β methylcholine)

		After shock											
Date	Patient	Before shock			After 1-3 min			After 15 min			After 60-90 min		
		ACh	BCh	MCh	ACh	BCh	MCh	ACh	BCh	MCh	ACh	BCh	MCh
12 i. 48	H.K.K	3518	1362	70	4100	1537	67	3694	1332	62	—	—	—
19 i. 48		3538	1443	75	3813	1774	74	3450	1312	41	—	—	—
26 i. 48		3688	1644	56	3901	1672	51	3682	1708	65	—	—	—
19 i. 48	D.R.D	2107	939	63	2360	1097	53	2163	1093	42	—	—	—
26 i. 48		2015	908	44	2232	1065	41	2264	1081	54	—	—	—
23 ii. 48		2391	1133	34	2665	1300	20	2478	1327	32	2408	1286	47
21 vi. 48		—	1080	27	—	—	—	—	—	—	—	—	—
3 v. 48	W	—	1186	42	—	1180	39	—	—	—	—	—	—
10 vi. 48		—	1162	35	—	1195	34	—	1142	32	—	1265	34
3 v. 48	R.T	—	1522	44	—	1583	41	—	1479	39	—	1355	42
10 v. 48		—	1242	37	—	1280	32	—	1290	35	—	1292	41
24 v. 48		—	1210	34	—	1346	36	—	1232	40	—	—	38
19 i. 48	G.K.W	3349	1414	66	3593	1350	98	3488	1456	80	—	—	—
26 i. 48		3395	1443	57	3595	1637	70	3195	1356	55	—	—	—
2 ii. 48		2962	1188	42	3625	—	61	3355	1374	51	—	—	—

special interest in view of the recent reports from the Maudsley Hospital in London of the experimental treatment of psychotic illness and depression with anticholinesterase substances (Nevin & Rowntree, 1948)

The abnormally high serum true cholinesterase in more than half the chronic psychotic patients may also be explained on a basis of supply and demand. Comparatively slight activity of nerve cells may call for the production of correspondingly little acetylcholine, while at the same time organs responsible for the production of cholinesterase may produce a minimum amount of the enzyme, which, however, is not required. It may be postulated that this high blood cholinesterase is an expression of a disturbed balance, but it would be premature to say as yet that this is characteristic of a psychosis, but may be an effect of age, more pronounced in psychotic than in normal individuals.

The mental defectives, being both young and not psychotic, can be imagined to have a neural equilibrium more resembling normal, and, as the cholinesterase level does not seem to be related to brain size or quantity of neurons, it is reasonable to expect that the serum cholinesterase would be within the normal range, which has been found to be the case.

The complete disappearance of cholinesterase in some of the oldest patients is probably due to the cessation of the production of an enzyme which has not been required for a long period, or to a general atrophy of the centres of production.

The remarkable absence of correlation between true cholinesterase in the serum and in the cerebrospinal fluid cannot be explained at present.

SUMMARY

1 Of fifty five aged male psychotics studied, thirty one had a high, thirteen a normal and eleven a low serum true cholinesterase.

2 Of sixteen mental defectives investigated fifteen gave values within the normal range. A low serum or cerebrospinal fluid cholinesterase level was not related to brain size.

3 No correlation could be observed between true and pseudo cholinesterase levels, or between serum and cerebrospinal fluid cholinesterase levels.

4 The significance of the true cholinesterase levels in the various groups is discussed.

5 From 1 to 90 min after electroconvulsive treatment the serum cholinesterase level may be raised, lowered or unchanged, but after repeated shocks there is a decrease in this level.

6 It is suggested that the changes in true cholinesterase levels in electroconvulsion treatment cases may imply a regulatory mechanism in which (1) cholinesterase is at once used by the central nervous system to break down acetylcholine, and therefore disappears from the blood, and (2) new cholinesterase is produced to enter the blood stream.

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Amino-acid Decarboxylases of Rat Liver

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(Received 22 April 1949)

Two amino acid decarboxylases are known to occur in the rat's liver 3,4-dihydroxyphenylalanine (DOPA) decarboxylase (Holtz & Credner, 1942), and cysteine acid decarboxylase (Blaschko, 1942a). Data on the activities of these two enzymes in liver extracts from normal rats were required, for comparison with observations on animals deficient in vitamin B₆ (Blaschko, Carter, O'Brien & Sloane Stanley, 1948). Differences were found in the cysteine acid decarboxylase activities of liver extracts from male and female rats, the present paper contains an analysis of these findings.

Page (1945) has not been able to find any DOPA decarboxylase activity in extracts from rat tissue, experiments reported below were carried out in which his findings were tested.

METHODS

The manometric measurement of decarboxylase activity followed closely the methods already described (Blaschko 1942a, b). Extracts were prepared as follows. The tissues were ground with sand in the cold for 5–10 min, until there was no further change in the appearance of the paste, ice cold 0.0675M sodium phosphate buffer of pH 7.4 (1 ml/g of tissue) was then added, and the mixture was centrifuged for 5 min. at a relative centrifugal force of about 800 g, to remove the sand and a few pieces of connective tissue and undamaged cells. The opaque viscous supernatant fluid was used. The enzymic activity is expressed as q_{CO_2} , i.e. as μ l CO₂ formed/mg fresh wt of tissue/hr. The substrate used is shown in parentheses, thus q_{CO_2} (DOPA) or q_{CO_2} (cysteine). In the calculation, the simplifying assumption was made that 1 ml of extract contained the enzymic activity from 500 mg of tissue.

For the manometric experiments, each flask contained 1.6 ml of extract 0.4 ml of either water or 0.01M substrate was added from the side bulb immediately after the initial readings. Readings were taken for 30 min, after which time the contents of each flask were acidified by adding 0.3 ml of 3N H₂SO₄ from the central well, in order to

determine the amount of CO₂ retained in the solution as bicarbonate. The substrates used were either L-3,4-dihydroxyphenylalanine or L cysteine acid. All experiments were carried out at 37.5° in an atmosphere of N₂. The enzymic activities of the extracts were calculated from the initial linear rates of formation of CO₂, corrected for retention.

Adult white rats of the Wistar strain were used throughout.

RESULTS

3,4-Dihydroxyphenylalanine decarboxylase activity of liver extracts

Extracts from eighteen normal rats' livers were tested, the results are given in Table 1. The mean value of q_{CO_2} (DOPA) was 0.69, there was no correlation of the enzymic activity with either sex or age of each rat. These experiments show that, under the

Table 1 3,4-Dihydroxyphenylalanine decarboxylase activities of rat livers

Body wt (g)	Sex	q_{CO_2} (DOPA) (μ l. CO /mg fresh liver/hr)
—*	—	0.438
—*	—	0.794
—	♂	0.575
155	—	0.820
165	—	0.820
170	+0	0.690
173	—	0.540
180	—	0.641
185	—	0.887
215	—	0.875
220	+0	0.820
230	—	0.706
250	—	0.643
270	♂	0.563
283	♂	0.590
305	♂	0.610
Mean		0.69
S.E. of mean		±0.03

* Each determination made with the pooled livers of two rats.

Table 2 *Cysteic acid decarboxylase activities of rat livers*

Group no Type of rats	q_{CO_2} (cysteic) (μ l CO_2 /mg fresh liver/hr)					
	I Normal ♀	II Normal ♂	III Castrated ♂	IV Spayed ♀	Va Spayed ♀, untreated	Vb Spayed ♀, + oestrone
	0 000	0 030	0 088	0 088	0 034	0 030
	0 031	0 053	0 098	0 097	0 040	0 054
	0 034	0 062	0 100	0 102	0 075	0 059
	0 044	0 088	0 100	0 135	0 088	0 060
	0 050	0 112	0 115	0 154	0 100	0 070
	0 050	0 119	0 125	0 169	0 138	0 080
	0 050	0 120	0 145	0 194	0 138	0 083
	0 050	0 125	0 152	0 231	0 145	0 094
	0 054	0 131	0 160	—	0 150	0 096
	0 056	0 137	0 169	—	0 153	0 150
	0 063	0 144	0 181	—	0 167	0 175
	0 063	0 144	0 212	—	—	—
	0 063	0 156	—	—	—	—
	0 063	0 163	—	—	—	—
	0 066	0 170	—	—	—	—
	0 073	0 172	—	—	—	—
	0 075	0 175	—	—	—	—
	0 081	0 200	—	—	—	—
	0 083	0 206	—	—	—	—
	0 090	0 206	—	—	—	—
	0 100	0 244	—	—	—	—
	0 119	0 250	—	—	—	—
	0 133	0 250	—	—	—	—
	0 240	0 260	—	—	—	—
	—	0 310	—	—	—	—
Mean	0 070	0 160	0 137	0 140	0 112	0 087
S E of mean (\pm)	0 008	0 013	0 011	0 018	0 014	0 008

conditions used, the enzymic activity of the rat's liver was easily measurable. A comparison with the data given by Blaschko (1942b) shows that the activity of liver extracts of other species (rhesus monkey, cat, dog, *Rana temporaria*) was much lower, the activity of guinea pig's liver extract was of the same order as that found in the present experiments with the rat.

The DOPA decarboxylase activity of two extracts of rat's kidney has also been determined, the values for q_{CO_2} (DOPA) found were 0 08 and 0 06.

Cysteic acid decarboxylase in rat's liver

Measurements of q_{CO_2} (cysteic) were made with extracts from the livers of ninety five animals. The results are given in Table 2, the mean value for each group and the standard error of each mean are also given.

The cysteic acid decarboxylase activities of extracts from different animals varied very widely. It was found that the mean enzymic activities of extracts from the livers of animals of opposite sex were different, this was shown by the experiments of groups I and II. The mean q_{CO_2} (cysteic) for twenty six female rats (group I) was 0 07, this was less than half the corresponding figure for twenty seven males (group II), which was 0 16. The probability that this difference arose by chance is less than 1 in 1000.

In order to analyse further the sex difference in

enzymic activity, experiments were carried out on castrated male rats and ovariectomized females. Twelve male rats were castrated, and the animals were killed 30 days after the operation (group III). The mean value of q_{CO_2} (cysteic) for liver extracts from these animals was 0 137, this value was not sufficiently different from that for the normal male rats of group II to suggest that castration had any significant effect.

The low cysteic acid decarboxylase activity of the extracts of female rats' livers was found to depend on the presence of the ovaries. Eight rats were ovariectomized and the enzymic activities of extracts of their livers were determined 25 days after the operation. These are the animals of group IV. The difference between the mean value for q_{CO_2} (cysteic) of 0 14 for this group and that for the normal male rats of group II was not significant ($P > 0 5$), on the other hand, the difference between this value and the mean for the normal females of group I is significant ($P \leq 0 01$).

In another series of experiments twenty two female rats were ovariectomized (group V). After a period of 3 weeks, eleven of these rats (subgroup Vb) were treated with oestrone, the remaining eleven animals (subgroup Va) served as untreated controls. Each rat of subgroup Vb received a subcutaneous injection of 2 μ g of oestrone in 0 2 ml of olive oil/100 g of body weight, every second or third

day This treatment was continued for a period of about 55 days, by the time of death, each animal had received between seventeen and twenty injections The mean value for the enzymic activity of liver extracts from the oestrone treated ovariectomized rats was 0.112, that for the untreated ovariectomized animals was 0.087 The difference between the mean values for enzymic activity in the two subgroups was small, the probability of the difference arising by chance being 1 in 8 Yet the higher value for the untreated animals differed significantly from that for normal females ($P = 0.02$), and the difference between the mean values for the oestrone-treated animals and for normal females was not significant

DISCUSSION

The observations on DOPA decarboxylase are in qualitative agreement with the statement of Holtz & Credner (1942) that extracts of rat's liver and kidney contain the enzyme, and they do not confirm the results of Page (1945), who failed to find the enzyme in the rat His figures for the enzymic activity in extracts of other tissues known to contain the enzyme are lower than those previously reported (Blaschko, 1942*b*)

In the present experiments, the DOPA decarboxylase activities of rat kidney extracts were found to be much lower than those for extracts of rat liver This is interesting, as in all other mammalian species hitherto examined the kidney has been the organ yielding extracts of the highest activity Quantitatively, the present data differ from those of Holtz & Credner (1942), who found a higher activity in rat kidney extracts This difference is probably due to the different technique employed by these authors, who used more dilute tissue extracts, made with a buffer of pH 6.5 At this pH the enzymic reaction does not run to completion, in fact, the reaction comes to a standstill so quickly that it seems doubtful whether the method is useful for the accurate measurement of enzymic activity In the present experiments, the pH of the buffer used was 7.4, and the extracts were concentrated, the reaction was usually followed to completion and was found to be approximately quantitative

The experiments on cysteic acid decarboxylase may explain the apparent contradiction in the results of Blaschko (1942*a*) and Medes & Floyd (1942) The latter authors were not able to demonstrate any cysteic acid decarboxylase activity in the rat's liver The present experiments have shown that the mean enzymic activity in liver extracts from female rats is low, in some animals the activity was very low or almost absent If Medes & Floyd (1942) used female rats for their experiments, this might account for their failure to demonstrate the presence of the enzyme

The observations on the ovariectomized animals indicate that the low enzymic activity in liver extracts from the female rats depended on the presence of the ovaries, both series of ovariectomized animals had a high mean cysteic acid decarboxylase activity The lowered values in the animals treated with oestrone suggest that the presence of this hormone in the normal female is connected with the low enzymic activity

The sex difference observed was unexpected, as there is no obvious connexion between the ovaries and the reaction catalysed by cysteic acid decarboxylase The product of the reaction is taurine Taurine occurs in the body as a constituent of taurocholic acid, and it seems possible that the activity of the enzyme is linked up with the biosynthesis of taurocholic acid There is good evidence that the bile salts are concerned with the emulsification and absorption of fat in the intestine (see Frazer, 1946) It is therefore possible that the rate of absorption of fat in the rat's gut is limited, through the rate of supply of taurocholic acid, by the cysteic acid decarboxylase activity of the animal's liver

Sex differences in fat metabolism have been described by Burn & Ling (1934), who found that in rats on a diet rich in butter the injection of anterior pituitary extract caused ketosis in some of the females, but not in males Pregnant animals fed on the butter diet developed an intense ketosis just before parturition Furthermore, it was found by György, Rose & Shipley (1947) that ovariectomized female rats, fed on a diet rich in fat, developed fatty livers, the deposition of fat in the livers of these animals could be prevented by the administration of methionine, together with oestrone, but not by methionine alone

Together with the present findings, these observations raise the question whether the female rat, as a result of the low cysteic acid decarboxylase activity of its liver, does normally have a low rate of fat absorption Ovariectomy, or other factors raising the cysteic acid decarboxylase activity of the liver, might be expected to increase the rate of fat absorption to a level at which the animal would develop ketosis or a fatty liver The effects of anterior pituitary extract, pregnancy, and ovariectomy on the rate of absorption of fat in the gut and on the cysteic acid decarboxylase activity of the liver of the female rat could be tested experimentally

It has also been reported that the 'non specific cholinesterase' of rat liver is distributed unevenly between the sexes, extracts from the livers of females have a higher activity, which is lowered by ovariectomy and raised again by oestrogen treatment (Sawyer & Everett, 1947) The authors consider it possible that this enzyme is concerned in fat metabolism

SUMMARY

1 The 3,4-dihydroxyphenylalanine (DOPA) decarboxylase activities of extracts of rats' livers and kidneys, and the cysteine acid decarboxylase activities of extracts of rats' livers, have been measured

2 The DOPA decarboxylase activity of extracts of rat liver is comparatively high, being of the same order as that previously recorded for extracts of guinea pig liver. The rat is unusual in that the DOPA decarboxylase activity of an extract of its kidney

is much lower than that of its liver, the ratio is about 1/10

3 The mean cysteine acid decarboxylase activity of extracts of the livers of male rats is about twice that of females. This difference can be abolished by the spaying of the females, but not by the castration of the males, it is partly restored by the injection of oestrone into the spayed females

I wish to thank Prof J H Burn, FRS, and Dr H Blaschko for their help and encouragement, also Dr Blaschko and Dr J M Walker for performing operations on some of the rats. This work was done during the tenure of a Medical Research Council Studentship

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Methods for the Determination of *N*-Methyl-2-pyridone-5-carboxylamide and of *N*-Methyl-2-pyridone-3-carboxylamide in Human Urine

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(Received 30 May 1949)

Knox & Grossman (1946, 1947) showed that *N*-methylnicotinamide can be oxidized by an enzyme in rabbit liver to *N*-methyl 2-pyridone 5-carboxylamide and isolated the latter compound from human urine after the ingestion of doses of 0.6–0.9 g of nicotinamide per day. The conversion of part of the *N*-methylnicotinamide formed in the body into the pyridone may explain why, in the past, it has been possible to account for only a small proportion of a dose of nicotinamide in the urine in the form of then known metabolites of nicotinic acid, and why the urinary excretion of *N*-methylnicotinamide shows little correlation with the dietary intake of nicotinic acid (Perlzweig & Huff, 1945; Knox & Grossman, 1946), but before the full significance of this new metabolite of nicotinic acid can be elucidated, an accurate method for its determination in urine is required.

Holman & Wiegand (1948) synthesized *N*-methyl 2-pyridone 5-carboxylamide by methylation and oxidation of nicotinic acid, and conversion of the

resulting *N*-methyl 2-pyridone 5-carboxylic acid to the corresponding amide. They found that the methylation and oxidation of nicotinamide gave *N*-methyl 2-pyridone 3-carboxylamide. The work of Knox & Grossman gave no evidence that the latter compound is excreted in human urine after administration of nicotinamide, but further information on this point is desirable.

Since pure specimens of both the isomeric amides were available, a study of their properties was made, in an effort to find reactions which could be used for their determination in urine. It was observed that the 2,3-amide could be nitrated at room temperature in the presence of concentrated sulphuric acid, and that the resulting nitro compound gave an intense and reasonably stable colour when made alkaline with sodium hydroxide. The 2,5-amide also could be nitrated, but only by the application of heat, its nitration product gave a yellow colour with weak alkalis as well as with sodium hydroxide. The colour faded rapidly in the presence of sodium hydroxide,

but was reasonably stable in the presence of weak alkalis. The compounds could be estimated colorimetrically by these reactions.

Before the methods could be applied to urine it was necessary to remove other substances which are capable either of being nitrated to derivatives which are coloured in alkaline solution (e.g. derivatives of phenol, imidazole, purine and pyrimidine), or of substances interfering in other ways, e.g. by charring in concentrated sulphuric acid. It was found possible to separate pyridones either present in, or added to, urine from almost all extraneous matter by making use of their slight solubility in chloroform and of their capacity to be adsorbed by Lloyd's reagent. By the application of these principles, procedures were worked out for the determination of both amides in human urine.

METHODS

Reagents

5N H_2SO_4 , 0.1N H_2SO_4 , 0.4N NaOH in methanol (diluted from saturated aqueous solution of NaOH with A.R. methanol), methanol (A.R.), N NaOH, CHCl_3 (grade for anaesthesia), nitrating mixture (1 g pure KNO_3 dissolved by warming in 6 ml pure conc. H_2SO_4 , freshly prepared and cooled before use), 95% (v/v) ethanol, 15% (w/v) Na_2CO_3 , standard solutions of *N*-methyl 2-pyridone 5-carboxylamide and *N*-methyl 2-pyridone 5-carboxylamide (0.1 mg/ml), the solutions were stable for at least a month when kept in a refrigerator).

Procedure

Specimens of urine were collected every 24 hr. in 25 ml of a mixture of equal volumes of glacial acetic acid and toluene. If the total volume was <1500 ml the specimen was diluted with water to 2 l. Specimens were usually filtered immediately after collection and the filtrate analysed without delay. Samples stored in a refrigerator for 5–10 days and then filtered gave slightly lower results, apparently owing to loss by adsorption. In such cases it was necessary to dissolve the sediment as well as possible by warming and mixing, and to omit filtration.

Determination of

N-methyl 2-pyridone 5-carboxylamide

Adsorption on Lloyd's reagent With normal urines, 25 ml of the filtered sample were transferred to each of four 50 ml centrifuge tubes. If the pyridone content was exceptionally high, e.g. after the ingestion of a dose of nicotinamide, it was necessary to dilute the urine to a suitable extent before analysis. Two of the tubes were labelled *U* (unknown). To each of the remaining tubes was added 1 ml of the standard solution of *N*-methyl 2-pyridone 5-carboxylamide and the tubes were labelled *R* (recovery). From this stage onwards, until the final extraction with CHCl_3 had been completed, all four tubes were treated in exactly the same way. One ml of 5N H_2SO_4 was added to each tube, followed by 2 g Lloyd's reagent. The tube was stoppered, shaken for 5 min, centrifuged and the supernatant fluid discarded. To the Lloyd's reagent in the tube were added 25 ml of 0.1N H_2SO_4 and, after shaking and centrifuging, the supernatant

fluid was again discarded. To the Lloyd's reagent in the tube were added 10 ml 0.4N NaOH in methanol and, after shaking for 5 min and centrifuging, the supernatant fluid was transferred to a 150 ml glass stoppered extraction flask. The residue in the centrifuge tube was washed with 5 ml methanol and, after mixing and centrifuging, the washings were added to the contents of the flask. The extract and washings were neutralized with glacial acetic acid (0.15 ml) and evaporated in a boiling water bath to a volume of 1 ml, and then to dryness with the aid of suction, taking care to avoid loss by sputtering and to obtain uniform drying.

Extraction with chloroform Exactly 0.5 ml aqueous N NaOH and 25 ml CHCl_3 were added to the dry residue in the flask, which was stoppered (no grease) and shaken for 5 min. The mixture was filtered through a dry paper (Whatman no. 42, 12.5 cm), taking care to minimize evaporation, into a glass stoppered 50 ml measuring cylinder containing 10 ml of a dilute aqueous solution of acetic acid (2 ml glacial acetic acid/l.) until the total volume was exactly 30 ml. The contents of the measuring cylinder were shaken for 5 min and the layers allowed to separate.

Portions (5 ml) of the aqueous layer were transferred from each measuring cylinder to a 7 × 1 in Pyrex test tube and the appropriate tubes were marked *U* and *R*. The fluid remaining in the four measuring cylinders was combined, the layers were allowed to separate, and 5 ml of the aqueous layer transferred to a fifth test tube and marked *B* (blank). The contents of the five tubes were evaporated to dryness in a boiling water bath, the residue was perfectly white in colour.

Nitration Exactly 0.3 ml of nitrating mixture was added to the bottom of each tube which was shaken to dissolve the residue. The tubes marked *U* and *R* were heated for 1 hr in a boiling water bath and the tube marked *B* was left at room temperature for 1 hr.

Development of the colour This step was carried out under carefully controlled conditions. An apparatus was used consisting of an electrically driven stirrer with a blade which fitted closely into the bottom of a 7 × 1 in test tube, and three burettes so arranged that they could deliver water, 15% (w/v) Na_2CO_3 and ethanol, respectively, into the tube with continuous stirring. The stirrer was made by flattening the end of a glass rod and shaping to fit the tube. A freezing bath at -6° was prepared by insulating a wide mouthed vessel of about 1 l capacity with asbestos and filling with a solution of 100 g NaCl in 900 ml water. The temperature was reduced to -6° by the addition of powdered 'dry ice' and maintained by the occasional addition of a further spoonful of 'dry ice'.

After the nitration each tube was treated, in turn, as follows. The tube was clamped so that the stirrer fitted closely into its base. After switching on and ensuring that the 0.3 ml of nitrating mixture was being mixed thoroughly and that the stirrer speed was about 300 r.p.m., the freezing bath was raised until the tube was well immersed. After stirring for 0.5 min, 1 ml distilled water was added, drop by drop, over a period of approx. 1 min, followed by 5 ml 15% (w/v) Na_2CO_3 , drop by drop, over a period of 4–5 min. The freezing bath was lowered until the tube no longer dipped into it and 20 ml ethanol were run in during 1 min. The stirrer was switched off, the tube removed, and the contents transferred to a centrifuge tube and centrifuged for 10 min at 3000 r.p.m. to remove precipitated Na_2CO_3 , 20 ml of the supernatant fluid were pipetted into a flask

and diluted with 5 ml distilled water (to prevent the appearance of turbidity on standing)

The colour intensity of each solution was measured against the blank in a photometer at 450 m μ , using a 3 cm cell. The blank was colourless

Calculation of results *N* methyl 2-pyridone 5-carboxylamide (mg excreted/24 hr) = $U/(R - U) \times 0.1 \times V/v$, where U and R are the average optical densities of unknown and recovery respectively, V is total vol (ml) of 24 hr sample of urine after all dilutions have been made, and v is vol (ml.) of urine taken for analysis (25 ml.)

Determination of *N* methyl 2-pyridone-3 carboxylamide

The procedure was very similar to that described above. Except that 1 ml. of the standard solution of the 2 3 amide was used, the technique for the adsorption on Lloyd's reagent and for the extraction with CHCl₃ was identical in the two methods

After extracting with CHCl₃, 5 ml. of the aqueous layer were transferred from each measuring cylinder to a test tube and evaporated to dryness. A separate blank was not required. To each tube was added 0.3 ml nitrating mixture and the residue dissolved by rotating the tube. After standing for several minutes at room temperature, the contents were diluted and neutralized at -6°, as described above. After adding 20 ml. ethanol and centrifuging off the precipitated Na₂CO₃, 20 ml of the supernatant fluid were diluted with 5 ml distilled water. The optical density of the solution was read against distilled water at 420 m μ , using a 3 cm cell, before and after the addition of 0.05 ml saturated aqueous KOH. The 24 hr excretion in mg was calculated by the formula given for the 2 5-amide, taking U as the average difference between the optical density of the unknown before and after the addition of KOH, and R as the corresponding difference obtained with the recovery

RESULTS AND DISCUSSION

Accuracy of the methods

Under the conditions of nitration and colour development described above, the relationship between optical density and concentration of pyridone is linear (Fig 1). To obtain constant colour development with the 2 5 amide, care must be taken with the dilution and neutralization, since the colour intensity is greatly influenced by the conditions of temperature and pH. Sodium carbonate gave the best results of a number of weak alkalis tested. The effectiveness of the above technique probably lies in the fact that the carbon dioxide evolved during the reaction between the carbonate and the diluted acid mixture keeps the pH at the optimum value. In the case of the 2 3 amide these conditions are not so important.

With both isomers losses occur in the adsorption on Lloyd's reagent as well as in the extraction with chloroform, but the proportion of different added amounts of either the 2 5 or the 2 3 amide which is recovered by the present technique (about 30 %) is

constant for any particular sample of urine, provided that the analyses are carried out side by side in the same way, and that the amounts of added pyridone do not vary over too wide a range. There is a slight tendency for the percentage recovery to decrease as the amount of added pyridone is increased, but the effect is insignificant if the amount does not vary more than about threefold.

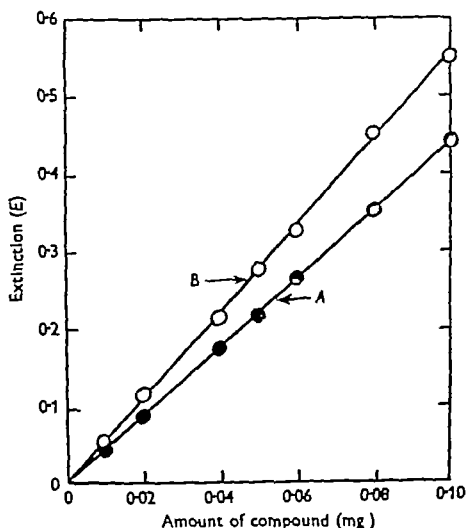


Fig 1 Typical standard curves for *N* methyl 2 pyridone 5-carboxylamide (A), and *N* methyl 2-pyridone-3-carboxylamide (B). A Lumetron colorimeter was used, with 1 cm cells.

The degree of precision of the method for the 2 5 amide, which has been examined in more detail than that for the 2 3 amide, should be adequate for most purposes. The average deviation between the optical densities of pairs of unknown solutions or recoveries has been found to be 2.5 %, and that between results for the pyridone content of urine calculated on the basis of one unknown and one recovery to be 10 %. Results calculated on average values for the optical density of two unknown solutions and two recoveries show a considerably lower degree of variability, of the order of 5 %.

The specificity of the two methods has been studied in considerable detail. Of a number of compounds which were nitrated in amounts of 0.5 mg, the following gave no colour when nitrated at room temperature or at 100°, and when made alkaline either with sodium carbonate or with potassium hydroxide: phenol, histamine, caffeine, xanthine, adenine, uric acid, guanine and thymine. Histidine, tyrosine and tyramine gave yellow colours but they were produced after nitration either at room temperature or 100°, and in the presence of either sodium carbonate or potassium hydroxide. Since

the blanks in the methods for both isomers are colourless, it is obvious that these compounds, if present in normal urine, are removed by the adsorption and extraction processes and do not interfere

In the method for the 2 3 amide a trace of brown colour of unknown origin develops on the addition of potassium hydroxide and gives apparent values of 0.1–1.5 mg/day for the urinary excretion of this isomer. Since the colour differs widely in spectral absorption properties from the yellow colour given by the 2 5 amide, the true amounts excreted must be less than these apparent values. The fact that no significant increase in the apparent values occurred after the ingestion of a large dose of nicotinamide (Table 1), strongly suggests that the 2 3 amide is not excreted in human urine.

Although the 2 5 amide gives no colour in the method for the 2 3 amide, the latter compound, if present in urine, would interfere in the method for the 2 5 amide, since, when nitrated at 100°, it behaves in a similar manner to the 2 5 amide, giving a yellow colour with sodium carbonate. Evidence has been provided above, however, that the 2 3 amide is not present in urine. Confirmatory evidence that the method for the 2 5 amide is specific for the latter compound is afforded by the fact that the yellow colours given by a large number of samples of urine, when read against the corresponding blanks, invariably showed the same spectral absorption properties as the yellow colour derived from pure *N*-methyl 2 pyridone 5 carboxylamide.

Although both the corresponding acids, namely, *N* methyl 2 pyridone 5 carboxylic acid and *N* methyl 2 pyridone 3 carboxylic acid can be nitrated and give the same respective colour reactions as the amides, they are not extracted from alkaline aqueous solution by chloroform, and therefore, if present in urine, would not be included in the result. A method for the determination of the 2 5 acid in urine is being

worked out and will be reported in a later communication. *N* ethyl 2 pyridone 3 carboxylamide, *N* benzyl 2 pyridone 3 carboxylamide, and *N*-benzyl-2 pyridone 5 carboxylic acid give similar colour reactions after nitration, but there is no reason to suspect the presence of these compounds in urine. *N*-methylnicotinamide gives no colour reaction. The specificity of the methods has been tested only with normal urines and with urines excreted by normal adults after administration of nicotinic acid and related compounds.

Application of the methods

In Table 1 are shown the results obtained by the present methods for the excretion of the 2 5 and the 2 3 amides by two healthy adult European subjects (no. 1, a male of 27 years, no. 2, a female of 36 years) on successive days before and after the oral administration of 500 mg of nicotinamide. The corresponding values for the urinary excretion of the total acid hydrolysable derivatives of nicotinic acid and of *N*-methylnicotinamide are also included in the table. The results show that the 2 3 amide is of no significance in nicotinic acid metabolism, but that the 2 5 amide is the most important metabolite of nicotinic acid which has been recognized up to the present time. Of the dose of 500 mg of nicotinamide, 47–57% was excreted as the 2 5 amide, while only 23–26% was accounted for in the form of all other known metabolites together.

The results suggest that the sum of the daily excretions of the 2 5 amide and of *N*-methylnicotinamide may possibly afford a much better index of nicotinic acid status than any criterion which has been suggested up to the present time. Further results on the excretion of the 2 5 amide and of other metabolites of nicotinic acid after the ingestion of various derivatives of nicotinic acid will be reported in a later communication.

Table 1 *Urinary excretion of various metabolites of nicotinic acid before and after oral administration of 500 mg of nicotinamide*

Subject no	Metabolite of nicotinic acid	Urinary excretion (mg /day)					Percentage of dose excreted in the urine
		Before dose		After dose			
		Day 1	Day 2	Day 1	Day 2	Day 3	
1	Total acid hydrolysable derivatives*	—	2.9	19.1	2.7	2.6	3
	<i>N</i> methyl nicotinamide†	—	5.7	127	9.2	11.5	23
	<i>N</i> methyl 2 pyridone 5 carboxylamide	—	7.5	256	36.6	19.9	47
	<i>N</i> methyl 2 pyridone 3 carboxylamide	—	<0.9	<1.6	<0.7	<0.6	0
					Total	73	
2	Total acid hydrolysable derivatives*	0.7	0.6	13.4	0.4	0.6	3
	<i>N</i> methyl nicotinamide†	1.8	2.3	110	5.5	4.2	20
	<i>N</i> methyl 2 pyridone 5 carboxylamide	6.7	6.5	295	61.3	15.6	57
	<i>N</i> methyl 2 pyridone 3 carboxylamide	<0.1	<0.7	<0.9	<0.7	<0.5	0
					Total	80	

* Determined by the method of Perlzweig, Levy & Sarett (1940)

† Determined by a modification of the method of Najjar & Wood (1940)

While this work was in progress an outline of a fluorimetric method for the determination of *N*-methyl 2 pyridone 5 carboxylamide in urine was reported by Rosen, Perlzweig & Handler (1948). The authors considered that their values for normal urines (5.0–13.2 mg/day) may have been somewhat low, but it is obvious that they are of the same order as those obtained by the present colorimetric method (see Table 1, and also Dean & Holman, 1949).

SUMMARY

1 Methods are described for the determination of *N*-methyl 2 pyridone 5 carboxylamide and *N*-methyl 2 pyridone 3 carboxylamide in human urine. The methods are based on the nitration of the compounds, after isolation from urine by adsorption on Lloyd's reagent followed by extraction with chloroform, and on their colorimetric determination by

means of the yellow colours given by the nitro derivatives in alkaline solution.

2 The accuracy and specificity of the methods is discussed.

3 Results are shown which indicate that the 2.3 amide is of no significance in nicotinic acid metabolism, but that the 2.5 amide is an important metabolite. The 2.5 amide was excreted by two normal adults in amounts of the order of 7 mg/24 hr. After the ingestion of 500 mg of nicotinamide, the urinary excretion of the 2.5 amide increased greatly, 47–57% of the dose being excreted in 72 hr as the latter compound.

4 It was possible to account for 73–80% of an orally administered dose of 500 mg of nicotinamide in the urine of two adult subjects as *N*-methyl 2 pyridone 5 carboxylamide, *N*-methylnicotinamide and the total acid hydrolysable derivatives of nicotinic acid.

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The Terminal Peptides of Insulin

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(Received 1 June 1949)

One of the outstanding problems of protein chemistry is the elucidation of the relative positions occupied by the amino acid residues in the protein molecule. A method for the identification of those terminal amino acids in which the α amino group is free has already been applied to several proteins (Sanger, 1945; Porter & Sanger, 1948). The protein is treated with 1,2,4-fluorodinitrobenzene (FDNB) and submitted to complete hydrolysis. Thereafter the *N*-2,4-dinitrophenyl (DNP) derivatives of the amino acids are separated from each other and quantitatively estimated. By this means insulin has been shown to contain glycine and phenylalanine in the terminal position, each present to the extent of two molecules per insulin molecule of molecular weight 12,000.

The method can be adapted in determining the sequence of amino acids which occupy positions near to the terminal amino acid residues. For this purpose

the protein is submitted to partial hydrolysis leading to the liberation of a series of *N*-2,4-dinitrophenyl (DNP) peptides. These differ from the other products of partial hydrolysis in that they are acids which can be extracted from acid solution by organic solvents and can thus be obtained relatively free from other unsubstituted peptides. The peptide mixture so produced is relatively simple and can be fractionated by chromatography on silica gel. Determination of the structure of the individual *N*-2,4-dinitrophenyl peptides then reveals the order of the amino acid residues in proximity to the free α amino groups. The present paper describes the results obtained by applying the method to insulin. The nature and order of the amino acid residues contiguous to the lysyl residues have also been determined.

Since insulin contains four free α amino groups, it was concluded that the molecule is built up of four

open polypeptide chains joined together by $-S-S-$ bridges. These $-S-S-$ bridges could be broken by oxidation (Sanger, 1949a), and on subsequent separation two main products could be obtained, an acidic fraction, *A*, which had only glycyl terminal residues, and a basic fraction, *B*, which had phenylalanyl terminal residues. For the identification of peptides described below these fractions have been used in place of the intact insulin so as to reduce the complexity of the peptide mixture dealt with.

METHODS

Materials

Insulin. The insulin used in all this work and in the earlier work (Sanger, 1945, 1949a) was crystalline bovine insulin having a S content of 3.2%. Fractions *A* and *B* of oxidized insulin and their DNP derivatives (referred to as DNP *A* and DNP *B* respectively) were prepared as previously described (Sanger, 1949a).

Silica gel. A number of workers who have used the silica gel solvent system procedure originally described have stated in print (Consden, Gordon, Martin & Synge, 1947; Phillips & Stephen, 1948), or in private communication, that they have experienced difficulty in that the bands have moved too rapidly down the columns. This was probably due to the properties of the silica used, although the factors enumerated below may also be contributory. (i) *Unwashed chloroform.* All $CHCl_3$ should be thoroughly washed with water to remove the ethanol added as a stabilizer. If unwashed $CHCl_3$ is used, all DNP amino acids will travel fast on a column of silica gel. (ii) *Overloading of the column.* If too high a concentration of DNP derivative is applied to a column it will form a fast-moving band which 'tails' badly. No more than about $2 \mu\text{mol}$. of each DNP derivative should be used on a column of diameter of 1 cm. to obtain satisfactory bands. Nevertheless, the optimum amount will depend on the particular experiment. To obtain the best separations, as are needed in the identification of a DNP amino acid by mixed chromatograms, the smallest amount (0.1 – $0.2 \mu\text{mol}$.) that can conveniently be seen on the column should be used. In quantitative estimations, a higher concentration minimizes the importance of incidental losses which are probably due to irreversible adsorption on the silica. The maximum concentration that will give a satisfactory separation should be used. In the case of the DNP peptides the limit is often set by the solubility of the derivative in the moving phase. (iii) *Water content of the silica.* The *R* values (Martin & Synge, 1941) of the derivatives vary considerably with the amount of water added to the silica, a high water content causing bands to move too rapidly. The actual capacity of the silica appears to vary from batch to batch, so that the optimal amount of water is best determined by experiments with known DNP derivatives.

All these factors apply also to the fractionation of peptides, as described below, and should be considered before discarding a gel as unsuitable for use with DNP derivatives. All gels prepared in this laboratory have been found to be satisfactory, though *R* values vary considerably from one batch to another. The gels were prepared by the method of Gordon, Martin & Synge (1943) from commercial waterglass obtained in 5 gallon drums from Joseph Crosfield

Ltd, Warrington. It would seem that the source of the waterglass is more important than the actual method of preparation.

Recently Middlebrook (1949) has worked out a method for fractionating the DNP amino acids using buffered columns (Moyle, Baldwin & Scarisbrick, 1948), which may be generally reproducible on any batch of gel. With peptides we have found that the bands tend to 'tail' rather badly on buffered columns, though they were useful for peptides that could not be dissolved easily in the solvents used on unbuffered columns, and satisfactory fractionations could often be carried out.

Fractionation of DNP peptides

The methods of fractionation of the DNP peptides are essentially the same as those used for the fractionation of DNP amino acids (Sanger, 1945; Porter & Sanger, 1948). For a preliminary extraction of the terminal DNP peptides from other amphoteric peptides the following procedure was used. The acidic solution of the partial hydrolysate was extracted three times with equal volumes of ethyl acetate, and the combined ethyl acetate solutions extracted three times with a 1% solution of $NaHCO_3$, each bicarbonate extract being washed twice in two separating funnels with ethyl acetate. In this way one has a simple three tube counter current procedure of the type used by Craig (1944). The three bicarbonate solutions were combined, acidified and extracted again using similar counter current procedure with ethyl acetate. This final ethyl acetate extract should contain only acidic substances. After taking to dryness the peptide mixture is fractionated on a suitable column. To describe the various solvent systems used the method and codes of Tristram (1946) are followed (Table 1). It has been

Table 1. *Solvent systems used on silica chromatograms*

Solvent systems	Stationary phase (ml./g. silica given in brackets)	Moving phase (saturated with stationary phase)
$CHCl_3$	Water (0.5)	$CHCl_3$
CB_1	Water (0.5)	$CHCl_3$ 1% (v/v) <i>n</i> butanol
CB_2	Water (0.5)	$CHCl_3$ 5% (v/v) <i>n</i> butanol
Etc		
Ea HCl	<i>n</i> HCl (0.5)	Ethyl acetate
Ea pH 7	3M phosphate buffer, pH 7 (0.6)	Ethyl acetate
$M_{6.5}$	<i>n</i> HCl (0.5)	Ether 66% (v/v) methyl ethyl ketone
$M_{6.5}$ pH 6.5	3M phosphate buffer, pH 6.5 (0.6)	Ether 66% (v/v) methyl ethyl ketone
Etc		

Acetone-cyclohexane prepared from 1 vol. water, 3 vol. acetone and 10 vol. cyclohexane (Sanger, 1945).

found that the most generally useful solvents for the separation of the acidic terminal DNP peptides are the $CHCl_3$, *n* butanol mixtures. The chromatographic purity of each band was usually established by running it on columns with other solvent systems.

Sometimes it is found that a soluble compound, after being passed through a column and taken to dryness, has

become apparently insoluble. This is believed to be due to the formation of salts, and the material can generally be brought into solution once again by adding a drop of conc HCl and a solvent in which it is readily soluble such as ether or ethyl acetate. Such a mixture is then taken to dryness and rapidly dissolved in the required solvent. This technique has also proved useful for dissolving DNP peptides (such as A5) which are themselves very insoluble.

It was found that the yellow peptides containing N^5 DNP lysine could be fractionated from one another very successfully on suitable silica columns, on which they form sharp well defined bands. Such bands, however, are often contaminated with other colourless peptides, which can be separated by adsorption chromatography on talc from acid solution. The DNP derivatives are adsorbed firmly on the talc, whereas the simple peptides and amino acids usually pass straight through the column.

To prepare the column, talc (5 g) was washed several times by decantation with N HCl. The suspension was then poured into a chromatogram tube of 1 cm diameter and allowed to settle under slight suction. The peptide mixture was then put on the column. The DNP derivatives were adsorbed in a band at the top. After washing with about 40 ml N HCl, they were eluted with a mixture of 4 parts ethanol and 1 part N HCl, in which they moved rapidly down the column. This technique could be applied to the separated DNP lysyl peptides or to the aqueous solution of the hydrolysate before fractionation.

When it was necessary to defer the working up of a DNP peptide fraction it was usually dissolved or suspended in dilute HCl and kept in the dark. No destruction of DNP derivatives preserved in this way has been observed.

Estimation of DNP peptides

The yellow DNP amino acids and peptides were estimated colorimetrically using the Beckman spectrophotometer. Fig 1 shows the spectral absorption curves for

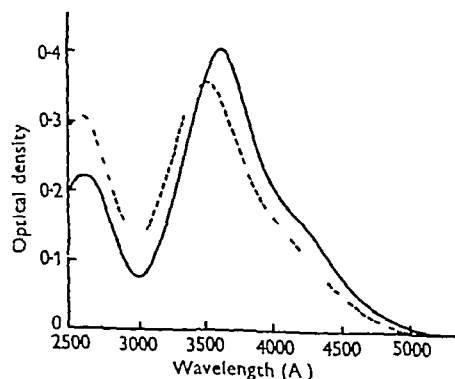


Fig 1 Spectral absorption curves of 23.7 μ M solutions of DNP phenylalanine (—) and DNP phenylalanyl valine (---) in 1% NaHCO_3 .

DNP phenylalanine and DNP phenylalanyl valine (prepared from DNP insulin, as described below) in 1% NaHCO_3 . It was found that the higher peptides of DNP phenylalanine (B3 and B4) gave absorption curves identical with that for DNP phenylalanyl valine at wavelengths

above 3000 A. To obtain these curves the concentration of DNP peptide was estimated from the amount of DNP phenylalanine produced on hydrolysis. The absorption curve for DNP glycine was almost identical with that of DNP phenylalanine, and the curve for DNP glycyglycine identical with that for DNP phenylalanyl valine. The DNP-glycyl peptides (A4 and A5) separated in the present work gave the same curve as DNP glycyglycine at wavelengths >3300 A. All these DNP derivatives give identical readings at 3500 A, and the estimations have been done at this wavelength. In most experiments readings were also taken at 3900 A and agreement between the results at the two wavelengths indicated the absence of other absorbing impurities. Table 2 shows the optical densities of 20 μ M solutions of the

Table 2 Optical densities of 20 μ M-solutions of DNP amino acids and peptides

DNP derivative	Solvent	Optical density (log I_0/I)	
		3500 A.	3900 A.
DNP glycine	1% NaHCO_3	0.309	0.210
DNP phenylalanine	1% NaHCO_3	0.313	0.214
DNP glycyglycine	1% NaHCO_3	0.316	0.173
DNP phenylalanyl valine	1% NaHCO_3	0.310	0.178
N^5 DNP lysine	N HCl	0.296	0.204
O DNP tyrosine	N HCl	0.058	0.0

different DNP derivatives. Beer's law is obeyed in solutions of concentrations $<50 \mu$ M. Standard curves for a number of other DNP amino acids in 1% NaHCO_3 have also been determined for a wavelength of 3500 A and have been found to differ only slightly from those of the above derivatives.

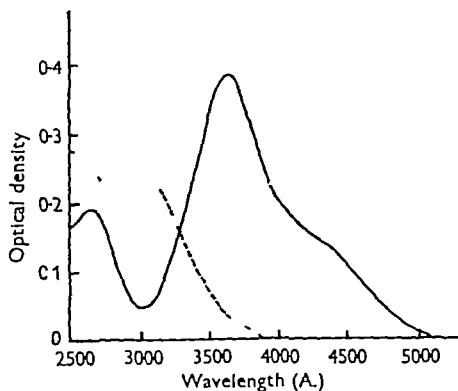


Fig 2 Spectral absorption curves of 22 μ M solutions of N^5 DNP lysine (—) and O DNP tyrosine (---) in N HCl.

The determinations of the peptides containing N^5 DNP-lysine were carried out in N HCl solutions, since N^5 DNP-lysine is unstable in alkaline solutions. The absorption curves for N^5 DNP lysine and O DNP tyrosine are shown in Fig 2. The curves for the lysyl peptides (L2, L3 and L4) described below were identical with that obtained for N^5 -DNP lysine at wavelengths >3300 A. In order to avoid

interference from *O* DNP tyrosine, estimations of *N*⁵ DNP lysine and its peptides were carried out using a wavelength of 3900 Å

Identification of amino-acids present in DNP peptides

The amino acids present in the DNP peptides were identified by paper chromatography (Consden, Gordon & Martin, 1944, 1947) After purification of the band on a suitable column, a sample containing 0.1–0.5 μmol peptide was taken to dryness in a 50 ml round bottomed flask, 5 drops of 5.7*N* HCl were added and the mixture refluxed for 1 min on a microflame to dissolve the peptide and wash down the sides of the flask The contents were sucked into a small capillary tube using a piece of valve rubber as described by Consden, Gordon & Martin (1947) The capillary was sealed off and incubated at 105° for 24 hr After cooling, the hydrolysate was transferred to a polythene strip and the HCl removed in a desiccator A suitable portion of the residue was then taken for identification of the amino acids by paper chromatography Since only a few amino acids were present in each peptide, it was best to use one dimensional chromatograms on which known amino acids were run parallel as controls In the case of fraction *A* all the amino acids present, except leucine and isoleucine, could be separated using the system phenol 0.3% NH₃ coal gas Leucine and isoleucine were differentiated using 1:1 *n*-butanol benzyl alcohol HCN All the amino acids from fraction *B* could be identified using the above mixtures and *n*-butanol acetic acid (Partridge, 1948) This latter solvent has been found to be very useful for amino acid identification and to replace collidine for most purposes, though the *R_F* values vary considerably with the age of the mixture, and it is always advisable to run known amino acids parallel to the unknowns *N*⁵ DNP lysine and *O* DNP tyrosine can also be identified using this solvent, whereas they both run with the solvent front on phenol chromatograms

If it is desired to identify the terminal residue as well as the other amino acids present, the above technique can be modified in the following way About 0.5 μmol of the DNP peptide is hydrolysed as before, if necessary, as in the case of DNP glycyl peptides, for a shorter period The hydrolysate is transferred to a test tube, 2 ml ether added and the tube shaken up The terminal DNP amino acid is extracted into the ether and can be identified on a suitable silica chromatogram The aqueous solution after evaporation of the ether on a water bath is used for the identification of the other amino acids present In the case of peptides from fraction *B* it was often necessary to determine whether the yellow compound present in the hydrolysate was DNP phenylalanine or *N*⁵ DNP lysine This was readily accomplished by decanting the ether extract of the hydrolysate into another test tube and shaking it with a few drops of NaHCO₃ solution *N*⁵ DNP lysine remains in the acid hydrolysate in the first test tube, whereas DNP phenylalanine passes into the NaHCO₃ solution in the other test tube

Estimation of amino acids present in DNP peptides

In order to determine the amount of each amino acid residue present in a peptide it was necessary to use an approximate micromethod, which could differentiate

between one, two or three amino acid residues per molecule Quantitative paper chromatography, as used in the 'spot dilution' method of Polson, Mosley & Wyckoff (1947), was found to be very suitable and sufficiently accurate for the purpose

After estimation of the DNP peptide in 1% NaHCO₃, a measured fraction (containing 0.5–1.0 μmol) was acidified and extracted three times with ethyl acetate, each extract being washed with water to remove salts The combined ethyl acetate solutions were taken to dryness *in vacuo* and the residue boiled under reflux with 5.7*N* HCl for a sufficient time to liberate all the terminal residue (2 hr in the case of DNP glycyl peptides and 4 hr for DNP phenylalanyl peptides) After cooling, the hydrolysate was extracted with ether, each extract being washed with water The DNP amino acid which was present in the ether extract was purified on a CHCl₃ column and estimated The combined aqueous solution and washings were taken to dryness and hydrolysed for a further 20 hr with 5.7*N* HCl Excess HCl was removed by repeated evaporation *in vacuo* and the residue made to a suitable volume Samples corresponding to various amounts between 0.05 and 0.005 μmol peptide were then applied to a one dimensional chromatogram and similar amounts of a standard solution of amino acids were applied alongside the unknowns After development of the chromatogram in a suitable solvent and treatment with ninhydrin the amount of each amino acid could be determined by comparing the colour and size of the spots In the case of the peptides of DNP glycine and DNP phenylalanine, phenol 0.3% NH₃ coal gas was a suitable solvent, and for the *N*⁵ DNP lysine peptides *n* butanol acetic acid was used

With certain peptides it was desirable to confirm the estimations by a more accurate method and it was found that, where only a few known amino acids were present, they could be estimated with reasonable accuracy by conversion to the DNP derivatives The following technique was used The aqueous hydrolysate from about 1.0 μmol of DNP peptide, after extraction of the terminal DNP amino acid, was taken to dryness and dissolved in 1 ml water NaHCO₃ (0.1 g) was added and a solution of 0.1 ml FDNB in 2 ml ethanol After shaking for 2 hr the solution was taken to dryness, a few drops conc HCl added and again taken to dryness The residue was extracted with CHCl₃, CB₁ or CB₂, according to the nature of the DNP amino acids present, and the extract put on the appropriate column Excess FDNB moved rapidly down these columns and was discarded, while the desired DNP amino acid bands were separated and estimated

RESULTS

Estimation of the terminal residues of fraction A and B of oxidized insulin

As a preliminary to the following work, and also to determine the average molecular weight of the peptide chains of fractions *A* and *B* of oxidized insulin it was necessary to estimate the terminal residues of these fractions as accurately as possible

The methods employed were those described in an earlier paper on insulin (Sanger, 1945), about 20 mg of material were used in each experiment, which was carried out in duplicate Since the main error is

probably due to the correction factor that has to be applied for the breakdown of the DNP amino acids during hydrolysis, the times of hydrolysis were kept short, and small amounts of peptides present were estimated separately. In the earlier work the amount of protein present in a DNP protein was estimated from the content of amide N. This required more material than was available, so the molecular weight of the peptide was calculated from the molecular weight of the DNP derivative by assuming that one DNP group (mol wt 167) is attached to every free amino group and to every tyrosine and histidine residue (Porter, unpublished work.) In the case of insulin this method gave the same results as the amide method.

Fraction A For the estimation of the DNP-glycine in DNP A a time of hydrolysis of 2 hr was used and the DNP glycine was purified on a CHCl_3 column. A small amount of a peptide band (probably A4) was adsorbed tightly at the top of the column. This was eluted with CB_{17} and estimated separately. The results are shown in Table 3. The

Table 3 *Terminal residues of fractions A and B*

(Results corrected for breakdown during hydrolysis)

Fraction	$\mu\text{mol. DNP}$ amino acid/ 100 mg DNP derivative	Mol. wt of DNP derivative
A DNP glycine	27	—
DNP glycyI peptide	2	—
Total	29	3450
B DNP phenylalanine	17	—
DNP phenylalanyl peptides	1.5	—
Total	18.5	5100
DNP glycine	1	—
N^5 DNP lysine	19.5	4800

molecular weight obtained for DNP A is 3450. Fraction A contains one free amino group and two tyrosine residues. It has no lysine or histidine (Sanger, 1949a), so that the DNP A should contain three DNP groups, giving an additional molecular weight of 500. The molecular weight of the average chain in fraction A is thus 2950, which agrees well with the value of 2900 determined in the ultracentrifuge (Gutfreund & Ogston, 1949). This value corresponds to a molecular weight of 2750 for the individual chains in the intact insulin, the difference being due to the oxygen taken up during the treatment with performic acid.

Fraction B DNP B was hydrolysed for 4 hr in boiling 5.7N HCl and the hydrolysate extracted with ether. The ether extract was used for the estimation of DNP phenylalanine and the aqueous solution for N^5 DNP lysine. The ethereal solution was taken to dryness and the residue put on a CHCl_3 column. Three bands were obtained. The main DNP

phenylalanine band was estimated directly. This was followed by a fainter band containing peptide B2 and a small amount of DNP glycine, which is usually present in preparations of fraction B. These two were separated on an acetone-cyclohexane column. There was also a faint band remaining at the top of the CHCl_3 column, which consisted of higher DNP phenylalanyl peptides and could be eluted with CB_{17} .

The aqueous solution, after extraction with ether, was hydrolysed for a further 20 hr and the N^5 DNP-lysine purified on an M_{88} column and estimated.

The results for DNP B, set out in Table 3, lead to an average molecular weight of 4950 on the assumption that the small amount of DNP glycyI peptide has the same molecular weight as DNP A. Fraction B contains two free amino groups, three tyrosine residues (estimated by difference between the tyrosine contents of insulin and fraction A) and two histidine residues (estimated directly on fraction B using histidine decarboxylase, Gale, 1945). DNP B should therefore contain seven DNP groups and the molecular weight of fraction B would be 3800, and of the intact chains in insulin 3700. The molecular weight as determined in the ultracentrifuge was 7000 (Gutfreund & Ogston, 1949), so that it would appear that either two phenylalanyl peptide chains are joined together by some non oxidizable cross linkage or that in solutions of fraction B aggregates are formed containing two molecules. Since non oxidizable cross linkages are unknown, while aggregation reactions of insulin are well known (summarized by Sanger, 1949b), the latter explanation would seem the more probable.

Isolation of DNP phenylalanyl valine from DNP insulin

When DNP insulin was hydrolysed for 2 hr with boiling 5.7N HCl, a peptide band was present that moved at the same rate as DNP glycine on a CHCl_3 column, but could be separated using an acetone-cyclohexane column. In the experiment described by Sanger (1945), in which the DNP amino acids were isolated from 1.03 g DNP insulin, the DNP glycine and the peptide were fractionated and the solution of the peptide in acetone-cyclohexane allowed to stand overnight. It crystallized as needles as the acetone evaporated (yield 8.1 mg).

A sample was hydrolysed and DNP phenylalanine identified as the terminal residue. Paper chromatography showed that valine and no other amino acid was present. By quantitative paper chromatography it was shown that there was one residue of valine per molecule of peptide and this was confirmed by estimation as the DNP derivative after purification on a CHCl_3 column (Found 3.3% valine N as percentage of peptide, calc for DNP phenylalanyl-

valine, 3 2, for DNP phenylalanyl valyl valine, 5 3 %) The only possible structure for this peptide is therefore DNP-phenylalanyl valine

Identification of DNP peptides from fraction B

In the initial experiments, DNP B was hydrolysed for 8 days with conc HCl at 37°

DNP-phenylalanyl peptides The material extracted from the hydrolysate by ethyl acetate was first put through a CB₁₇ column Most of the colour travelled fast and only a few faint bands could be distinguished on the column These were not investigated in detail The fast moving material was then fractionated on a CB₅ column Three main bands were formed, B3, B4 and a band that moved fast, there were also a number of fainter bands moving more slowly than B4 and a fairly strong one that remained at the top of the column and could be eluted with CB₁₇ Bands B3 and B4 were shown to be homogeneous on Ea pH 7 columns (*R* = 0 25 and 0 1 respectively) The material that moved fast in CB₅ consisted of DNP phenylalanine, DNP-phenylalanyl-valine (B2) and a small amount of DNP glycine These were separated using a CHCl₃ column, and the second band refractionated on acetone-cyclohexane In some experiments a band was present that was adsorbed at the top of the CHCl₃ column and usually split into a number of faint bands on development with CB₁ It contained a rather complex mixture of amino acids These bands were believed to be due to some secondary products (possibly esters), formed from the DNP peptides during the fractionation They did not occur in most experiments, and the same phenomenon was sometimes observed with DNP A

Table 4 *Peptides containing DNP phenylalanine*

Peptide	Column used for separation	<i>R</i> value	Amino acids present
B1	CHCl ₃	0 4	None
B2	CHCl ₃	0 2	Valine
	Acetone cyclohexane	0 7	
B3	CB ₅	0 6	Valine, aspartic acid
B4	CB ₅	0 3	Valine, aspartic acid, glutamic acid

The amino acids identified in the main fractions are shown in Table 4 In each case the terminal residue was DNP-phenylalanine, and quantitative paper chromatography showed that the amino acids

were present in equimolecular proportions in all the peptides The regularly increasing complexity of these peptides suggested that they were all derived from the same peptide chain containing the terminal sequence DNP-phenylalanyl-valyl aspartyl-glutamic acid, and this was confirmed by the following partial hydrolysis experiments

Peptide B3 (1 5 μmol) was boiled under reflux with 5 7*N* HCl for 1 hr and the degradation products studied chromatographically The results are shown in Table 5 The only products of hydrolysis were DNP phenylalanine and DNP-phenylalanyl-valine, which confirms the structure DNP phenylalanyl-valyl aspartic acid

Table 5 *Partial hydrolysis of peptide B3*

Peptide	Column used for separation	<i>R</i> value	Amino acids present
B3 1	CHCl ₃	0 4	None
B3 2	CHCl ₃	0 2	Valine
	Acetone-cyclohexane	0 7	
B3 3	CB ₅	0 6	Valine, aspartic acid

The partial hydrolysis of peptide B4 was carried out for 14 days at 37° in conc HCl All the bands B1, B2, B3 and unchanged B4 were identified and shown to contain the same amino acids as in Table 4 Since B4 gives rise to peptide B3, its structure can only be DNP phenylalanyl valyl aspartyl glutamic acid

Peptides containing N⁶-DNP lysine After extraction with ethyl acetate the aqueous solution from the hydrolysate of DNP B was taken to dryness and fractionated on an M₆₆ column Three main bands were formed, and the faster one was refractionated on M₆₆ pH 6 5 to give two bands (L1, L2) There was also a fainter fast band that appeared to consist of higher peptides of N⁵ DNP lysine Band L2 was shown to be homogeneous on Ea-HCl (*R* = 0 2) and CB₃₀ (*R* = 0 15) columns, and bands L3 and L4 were homogeneous on CB₃₀ (*R* = 0 07 and 0 04 respectively) Each peptide was purified by chromatography on talc, and the amino acids identified and shown to be present in equimolecular amounts The results are shown in Table 6 The terminal residues were determined by deamination with nitrosyl chloride (Consden, Gordon & Martin, 1947), and confirmed by the DNP technique It was found that the latter method gave a rather more clear cut result, whereas the former was much simpler to carry out In a preliminary report of this work

Table 6 *Peptides containing N⁶-DNP lysine*

Peptide	<i>R</i> values		Amino acids present	Terminal residue
	On M ₆₆	On M ₆₆ pH 6 5		
L1	0 6	0 3	N ⁵ DNP lysine	—
L2	0 6	0 1	N ⁵ DNP lysine, alanine	N ⁵ DNP lysine
L3	0 2	—	N ⁵ DNP lysine, threonine, proline	Threonine
L4	0 13	—	N ⁵ DNP lysine, threonine, proline, alanine	Threonine

(Sanger, 1948) the peptide *L4* was described as threonyl (*N*⁵ DNP) lysyl alanine. The presence of proline had been overlooked in the paper chromatography, the yellow spot being confused with that due to *N*⁵-DNP lysine. It can, however, be clearly detected if the solvent *n* butanol acetic acid is used.

The results in Table 6 show that peptide *L2* is *N*⁵ DNP lysyl alanine. Peptides *L3* and *L4* were hydrolysed for 11 days with conc HCl at 37°. The hydrolysate of *L3* contained only the bands *L1* and *L3*, and a faint band that moved at the same rate as *L2* on *M*₈₀ pH 6.5 and contained *N*⁵ DNP lysine and proline. From peptide *L4* all the bands *L1*, *L2*, *L3* and *L4* were obtained and were shown to contain the expected amino acids. Since *L4* has a terminal threonyl residue and gives rise to *N*⁵ DNP-lysyl alanine (*L2*) and a peptide (*L3*), containing threonine, proline and DNP-lysine, the only possible structure that can be assigned to it is threonyl-prolyl (*N*⁵-DNP-) lysyl alanine, and *L3* must be threonyl prolyl-(*N*⁵ DNP) lysine.

Estimation of peptides containing DNP phenylalanine and N⁵ DNP lysine in insulin

Having established the presence of the two peptide sequences, phenylalanyl valyl aspartyl glutamic acid and threonyl prolyl-lysyl alanine, in fraction *B* of the oxidized insulin, the next problem was to estimate the yields of the various peptides from insulin, and to discover what proportion of the lysyl residues and the terminal phenylalanyl residues of insulin was present in these peptide sequences. When DNP-insulin was hydrolysed for 8 days with conc HCl no peptides could be detected other than those which were obtained from DNP-*B* and very small amounts of DNP glycyl peptides which did not interfere with the estimation of peptides containing DNP phenylalanine and *N*⁵ DNP lysine. Almost all the terminal glycine was present as DNP glycine itself, owing to the great lability of the glycyl peptide bond in strong acid.

About 100 mg DNP insulin were hydrolysed for 8 days at 37° with conc HCl, the products fractionated as previously described for DNP-*B* and estimated colorimetrically. Peptides *B5*, *B6* and *B7* were obtained from bands that moved slower than *B4* on CB₅. It was shown that each gave rise to band *B4* on partial hydrolysis. *B8* was composed of a number of faint bands that moved slowly on CB₁₇. These were eluted from the column with 80% (v/v) acetone, hydrolysed and the DNP phenylalanine estimated after purification on a CHCl₃ column. The yields of the various peptides are shown in Table 7, expressed as moles of peptide as a percentage of the total DNP phenylalanine in the original DNP-insulin, assuming there are exactly two phenylalanyl terminal residues per insulin molecule of molecular weight 12,000. It can be seen that 92% of the

total DNP phenylalanine is accounted for as compounds that fit into the peptide sequence DNP-phenylalanyl valyl aspartyl-glutamic acid, and it would seem evident that in fact all the terminal phenylalanyl residues are present in this sequence. An actual estimation of the amount of the peptide *B4* in DNP insulin can, however, be obtained by studying the hydrolysis of the peptide itself under

Table 7 *Yields of DNP phenylalanyl peptides from an 8-day hydrolysate of DNP insulin*

(Results expressed as mol. of DNP peptide as percentage of the total terminal phenylalanyl residues)

Peptide	Yield (%)
<i>B1</i> DNP phenylalanine	13
<i>B2</i> DNP phenylalanyl valine	16
<i>B3</i> DNP phenylalanyl valyl aspartic acid	13
<i>B4</i> DNP phenylalanyl valyl aspartyl glutamic acid	30
Total known peptides (<i>B2</i> , <i>B3</i> , <i>B4</i>)	59
<i>B5</i>	6
<i>B6</i>	4
<i>B7</i>	10
Total higher peptides from the same sequence	20
<i>B8</i> Unknown mixture of peptides	6
Total	98

Table 8 *Yields of DNP peptides from an 8-day hydrolysate of peptide B4*

Peptide	Yield from <i>B4</i> (%)	Yield from DNP insulin (%)
<i>B1</i>	13	13
<i>B2</i>	14	16
<i>B3</i>	12	13
<i>B4</i>	55	50-56

the conditions that were used for hydrolysing the DNP-insulin. Thus, if all the DNP phenylalanine in DNP insulin is combined in the single tetrapeptide sequence, then the partial hydrolysis of peptide *B4* should give the same products in the same proportions as are obtained from DNP insulin. This assumes that the stability of the various peptide bonds is the same whether the DNP peptide is free or combined in the protein. While this may not be completely true it is probably a safe approximation. Table 8 shows the yields of DNP peptides from peptide *B4* (2 μmol) that had been hydrolysed for 8 days in conc HCl at 37°, compared with the yields from DNP insulin. The figure for the amount of *B4* obtained from DNP insulin includes higher peptides containing this sequence. It can be seen that within the limits of the methods the two sets of figures agree. The degree of accuracy of these results probably indicates that not more than about 10% of the terminal phenylalanine of insulin is present in sequences other than that detected here. Thus, not

only do these results prove that both the phenyl alanine terminal residues in the insulin molecule of molecular weight 12,000 are combined in the same tetrapeptide sequence, but they do justify the assumption of this value for the molecular weight

Table 9 *Yields of peptides containing N⁵ DNP lysine from an 8-day hydrolysate of DNP insulin*

(Results expressed as mol. of peptide as percentage of the total lysine)

Peptide	Yield from DNP insulin (%)	Yield from L4 (%)
L1 N ⁵ DNP lysine	14	14
L2 N ⁵ DNP lysyl alanine	19	23
L3 Threonyl prolyl (N ⁵ DNP) lysine	32	32
L4 Threonyl prolyl (N ⁵ DNP) lysyl alanine	23	21
Total known peptides (L2, L3, L4)	74	
L5 Unknown	6	
Total	94	90

Table 9 shows the yields of the various peptides containing N⁵ DNP lysine obtained from DNP insulin and from a similar hydrolysate of peptide L4. L5 is the uninvestigated band that moved fast on an M₆₆ column. The N⁵ DNP lysine in it was estimated after complete hydrolysis. The yields of the peptides from DNP insulin make it very improbable that there is a second type of peptide containing lysine and agreement between the two sets of figures confirms this.

Identification of DNP glycyI peptides from fraction A

Hydrolysis in concentrated hydrochloric acid
Owing to the great lability to strong acid of the peptide bond involving the carboxyl group of the terminal glycyI residues, partial hydrolysates of DNP-A carried out in conc. HCl contained large amounts of DNP glycine and of long peptides that could not be investigated easily, and only poor yields of small peptides that could readily be studied. Nevertheless, it was possible to determine the structure of three of these small peptides, and as most of the work was carried out on such hydrolysates, the results are reported here, although much more satisfactory yields were obtained at a later stage when the hydrolysis was carried out in dilute acid.

DNP-A was hydrolysed for 24 hr with conc. HCl at 37°, and the material that was extracted with ethyl acetate first put on a CB₁₇ column. Besides the main fast moving band there were a number of fainter bands. The strongest of these, which travelled at a gradually decreasing rate down the column, gave rise to the amino acids leucine, serine, glutamic acid

and O DNP tyrosine on hydrolysis. A peptide containing these amino acids had been reported by Woolley (1948), and it was at first assumed that the two were the same (Sanger, 1948). On quantitative estimation, however, it was found that there were about five residues of each of the above amino acids per residue of DNP glycine, so that the fraction was a mixture of a small amount of a DNP-glycyI peptide (probably A5), which was responsible for the colour of the band on the column, and a large amount of a non terminal peptide containing the above four amino acids. The exact structure of this peptide has not yet been determined, but its presence does indicate that DNP peptides, separated by the above methods, may be contaminated by non terminal peptides.

The coloured material that moved fast on the CB₁₇ column was next put on a CB₅ column. This separated a band (A4) which was run out by development with CB₈. The fast moving material from this column was fractionated on a CHCl₃ column. A very strong DNP glycine band (A1) was present, and this was followed by two peptide bands (A2 and A3) which were developed with CB_{0.5} and CB₁. Band A2 was relatively faint and sometimes difficult to detect. Bands A3 and A4 were homogeneous on Ea pH 7 columns (*R* = 0.4 and 0.1 respectively) and band A4 was homogeneous on M₆₆ pH 7 (*R* = 0.4) and M₆₆ pH 8 (*R* = 0.07).

Table 10 *Peptides of DNP glycine*

Peptide	Column used for separation	<i>R</i> value	Amino acids present
A1*	CHCl ₃	0.2	—
A2	CHCl ₃	0.06	Isoleucine
	CB _{0.5}	0.2	
	CB ₁	0.6	
A3	CHCl ₃	0.03	Isoleucine, valine
	CB _{0.5}	0.1	
	CB ₁	0.35	
A4	CB ₅	0.25	Isoleucine, valine, glutamic acid
	CB ₈	0.8	

* DNP glycine

Table 10 shows the amino acid composition of the three bands. In each case the terminal residue was DNP glycine. Estimation showed that the amino acids in A3 and A4 were present in equimolecular proportions to the DNP glycine. The composition of the peptides suggests that they are all derived from the same peptide chain containing the terminal sequence DNP glycyI isoleucyl valyl glutamic acid and that this was the structure of A4 was shown by partial hydrolysis. A sample was refluxed for 12 hr with 0.1N HCl. The hydrolysate contained the peptide bands A2 and A3 and isoleucine and valine were identified in the A3.

Hydrolysis in hydrochloric acid (0.1N) In an attempt to find a more specific method for hydro

lysing proteins, the action of boiling 0.1N HCl on DNP A was studied. It was found that the specificity of this hydrolytic agent was very different from that of concentrated HCl, and that the smaller DNP peptides were produced in much better yields. It seems probable that the hydrolysis of these oxidation products in dilute acid is analogous to the hydrolyses catalysed by long chain sulphonic acids (Stenhardt & Fugitt, 1942), the peptide chains containing $-SO_3H$ groups acting as long chain anions and catalysing a particular type of hydrolysis which is characterized by the increased lability for the amide groups and no doubt a corresponding difference in specificity towards other peptide bonds. That the $-SO_3H$ groups were catalysing the hydrolysis is indicated by the difference in the rate of hydrolysis of insulin and oxidized insulin illustrated in Fig. 3. No such difference was found when the hydrolyses were carried out in conc. HCl in which the $-SO_3H$ groups are uncharged. In these experiments the liberation of free amino groups was followed using the Van Slyke apparatus.

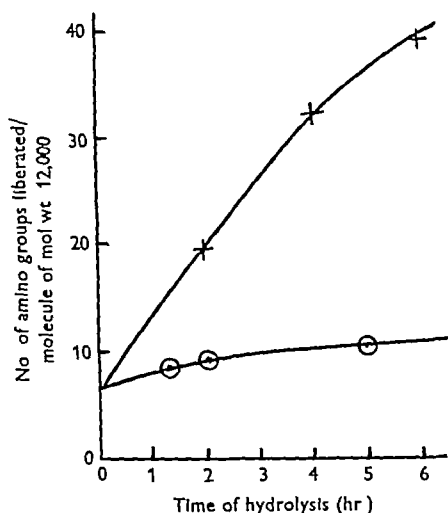


Fig. 3 Rate of hydrolysis of insulin (O) and oxidized insulin (+) in boiling 0.1N HCl

It should be noted that the formation of diketopiperazines in dilute acid has been demonstrated (Abderhalden & Komm, 1924), so their presence was a possibility in these experiments, though the temperature employed was probably too low. The DNP derivatives could not undergo any rearrangement of this type as they contain no free amino group, and there was no evidence of any contamination of the DNP peptide bands with diketopiperazines.

In a hydrolysate of DNP A that had been boiled under reflux for 12 hr with 0.1N HCl there was present, besides the three previously described peptides, considerable amounts of another peptide

(A 5) which moved with $R = 0.9$ on CB_{17} and was best purified on CB_{15} ($R \approx 0.4$), though it was extremely insoluble, and only faintly coloured bands could be obtained. On an Ea pH 6 column it gave an apparently homogeneous band with $R = 0.7$, though it 'tailed' rather badly on all buffered columns. This peptide contained DNP glycine and the amino acids isoleucine, valine and glutamic acid only. Quantitative paper chromatography showed that there were two residues of glutamic acid to every one residue of DNP glycine, isoleucine and valine, and this was confirmed by estimation of the glutamic acid as the DNP derivative. No amide group was present, since no NH_2 was formed on hydrolysing for 4 hr with boiling 2N HCl. Table 11 shows the results of an

Table 11 Partial hydrolysis products of peptide A 5

Peptide	Column used for separation	R value	Amino acids present	Yield of peptide (%)
A 5 1	$CHCl_3$	0.1	—	29
A 5 2	CB_1	0.6	—	4
A 5 3	CB_1	0.35	Isoleucine, valine	10
A 5 4	CB_5	0.25	Isoleucine, valine, glutamic acid	19
A 5 5	CB_{15}	0.4	Isoleucine, valine, glutamic acid	27

experiment in which A 5 was hydrolysed for 12 hr with boiling 0.1N HCl and the hydrolysis products investigated. It can be seen that it breaks down to give peptides A 2, A 3 and A 4, therefore the only possible structure is DNP glycyl isoleucyl valyl glutamyl glutamic acid.

Estimation of the DNP glycyl peptides from DNP insulin

Since DNP insulin is insoluble in 0.1N HCl it was not possible to obtain a significant yield of DNP peptides by hydrolysing it directly. However, if the insulin was first oxidized and then treated with FDNB, there was very little loss of material, and the DNP A present was largely soluble and gave good yields of the peptides.

In order to obtain the maximum possible yields of peptides at the expense of the yield of DNP glycine, the hydrolysis mixture was extracted from time to time during the course of hydrolysis, so that the larger extractable peptides (A 4 and A 5) were removed and not broken down to DNP glycine. Also unidentified fractions were hydrolysed further to give the known peptides.

As a preliminary, the breakdown of DNP B under these conditions was studied. Owing to its insolubility it was not extensively hydrolysed. The main breakdown products were peptide B 3 and a little B 2. There was also a small amount of an unidentified DNP phenylalanyl peptide that contaminated A 5.

Insulin (0.2 g) was oxidized with performic acid for 30 min (Sanger, 1949a). The product was precipitated with acetone and treated with FDNB for 2 hr in the usual manner. To remove excess FDNB about 50 ml ether and 5 ml water were added to the reaction mixture and, after shaking and allowing to settle, the ether layer was decanted and the aqueous layer and insoluble material were extracted twice more with ether in the same way. It was then acidified with HCl and taken to dryness *in vacuo*. Excess HCl was removed by repeated evaporation *in vacuo* with water, 50 ml 0.1N HCl were added and the mixture boiled under reflux for 3 hr. It was then cooled, and extracted twice with an equal volume of ethyl acetate. After removal of dissolved ethyl acetate from the aqueous layer by evaporation *in vacuo*, it was boiled for another 3 hr and again extracted. This process was repeated six times in all, making a total time of hydrolysis of 18 hr. The ethyl acetate solutions were taken to dryness after each extraction, and the residues stored with a little water. They were all combined and the DNP peptides purified by extraction into bicarbonate as previously described. About 3% of the DNP glycine was lost during this treatment. The final extract containing the terminal DNP peptides was taken to dryness, the residue dissolved in 80% ethanol and samples taken for the analyses. Several replicate analyses were performed, but not all peptides were estimated in each case.

Peptide A5 was separated on a CB₁₅ column. It was usually contaminated with a small amount of a DNP phenylalanyl peptide, which was estimated from the amount of DNP phenylalanine produced on hydrolysis of the fraction. Peptide A4 was fractionated on a CB₅ column and was free from any DNP-phenylalanyl peptides. Band B3 and a faint band B4 moved in front of the A4 band on this column, and there were traces of other unidentified DNP-phenylalanyl peptides moving more slowly. Pure bands, A2 and A3, were separated from a CHCl₃ column which was developed with CB₁. The DNP glycine fraction obtained from the CHCl₃ column contained peptide B2 which was separated on an acetone/cyclohexane column. The yields of the

various derivatives are shown in Table 12. The figures are given as percentages of the terminal glyceryl residues of the original insulin taken. The corresponding yields of the DNP-phenylalanyl peptides B1, B2, B3 and B4 were 3, 9, 20 and 3% respectively.

On the CB₁₅ column much colour was present in a number of slow moving bands which were eluted together from the column with 80% acetone and were designated fraction Y. This fraction contained about 20% of the DNP glycine as estimated after hydrolysis. A sample was hydrolysed for 12 hr with boiling 0.1N HCl, and the products estimated as for the main hydrolysate. It gave rise to the expected peptides showing that the DNP glycine present in it was largely in the form of higher peptides of the same sequence. The yields are shown in column 4 of Table 12.

Another 8% of the DNP glycine was in the insoluble residue (fraction R) from the original dilute acid hydrolysate. This was probably present as the DNP derivative of some incompletely oxidized insulin. In order to hydrolyse it further it was first brought into solution by incubating for 1 hr at 37° with conc HCl. After removal of the HCl *in vacuo*, the residue was hydrolysed for a further two periods of 12 hr with 0.1N HCl, and the peptides estimated as before. The yields are shown in column 5 of Table 12.

The total yields of peptides leave no doubt that both the terminal glyceryl residues are combined in the same terminal peptide sequence. It was not possible to determine the amount of peptides A4 or A5 directly by studying their breakdown under identical conditions of hydrolysis, as was done for peptides B4 and L4, since the type of hydrolysis used here could not be reproduced on a peptide. The unchanged peptide would be removed on each extraction with ethyl acetate, whereas long peptides from DNP-A remained in the aqueous solution and were hydrolysed further. It is also probable that the long chain charged polypeptides have a catalytic effect on the hydrolysis, which it would be difficult to reproduce exactly on a peptide. The sum of the yields of A4 and A5 (55%) does, however, give direct proof that over

Table 12 Yields of DNP glyceryl peptides from DNP insulin

(Results expressed as mol of peptide as percentage of the terminal glyceryl residues in the original insulin)

Peptide	Yield from main hydrolysate	Mean	Yield from fraction Y (%)	Yield from fraction R (%)	Total yield (%)
A1	17.5, 16	17	3.3	1.3	22
A2	1.4	1			
A3	4, 5	5	2.6	0.5	9
A4	11, 11	11	2.9	1.1	15
A5	37, 30, 38, 35	35	3.5	1.9	40
Total known peptides					64
Total					86

half of the terminal glycyl residues are in the form of the sequence glycyl isoleucyl valyl glutamic acid Table 11 shows that neither of these peptides is completely stable to this type of hydrolysis, so that the actual amount present must be considerably greater

DISCUSSION

It has been shown that both the terminal phenylalanyl residues are combined in the insulin molecule in the form of the single tetrapeptide sequence phenylalanyl valyl aspartyl glutamic acid, and that both the lysyl residues which are in the same chains are in the form of the sequence threonyl prolyl lysyl alanine. Also both the terminal glycyl residues of the other two chains are present in the pentapeptide sequence glycyl isoleucyl valyl glutamyl glutamic acid. The simplest explanation of these results is that the two phenylalanyl chains are identical, and that the two glycyl chains are identical. In the phenylalanyl chains, which contain about thirty amino acid residues, eight of the positions relative to the terminal residues and the lysyl residues are occupied by the same amino acids in both chains. It is, of course, possible that they might differ in the nature of one or a few residues in a position in the chain not investigated in this work. As nothing is known about the principles which govern the arrangement of amino acids along a peptide chain, such a possibility cannot be excluded. However, if one assumes that each position in the chain can be occupied by any of the sixteen different amino acids present in these chains, the chance that two different chains would contain the same terminal tetrapeptide is about 1 in 50,000. Certain limits are obviously imposed by the specificities of the mechanisms responsible for protein synthesis. Investigations of the free amino groups of a number of proteins by the DNP technique (summarized by Sanger, 1949c) have shown that the terminal position in the protein chains may be occupied by a variety of different amino acids. There appears to be no principle that defines the nature of the residue occupying this position in different proteins and it would seem probable that this would apply to other positions in the molecule. It would thus seem a reasonable conclusion from the above results that the insulin molecule is built up of two pairs of identical chains. However, if this were indeed the case, then the simplest asymmetric unit of insulin should have a molecular weight of 6000 and all the analytical figures for the amino acids should fit such a unit. The most recent data on the amino acid composition of insulin have been collected by Tristram (1949). While for most amino acids the number of residues per unit of molecular weight 6000 approximates to a whole number within the limits of the methods of assay, the values for proline and isoleucine would

indicate about 1.5 and 1.3 residues respectively. It is very unlikely that these estimations are more than 10% in error, so that they do cast considerable doubt on the above conclusion, and all that can be definitely stated is that the insulin molecule is built up of two pairs of very similar chains.

In contrast to the results of this work are the results obtained by Woolley (1949). Using a method similar in principle to that used here, he has reported the isolation of a number of peptides from a tryptic hydrolysate of DNP insulin, which he believed to be DNP glycyl peptides. He was unable, however, to demonstrate the presence of significant amounts of DNP glycine on hydrolysis of the peptides concerned, although it could be detected in a hydrolysate of DNP insulin. This and the results reported in the present paper would seem to indicate that the peptides isolated by Woolley were not DNP glycyl peptides. He used ethyl acetate and butanol extracts which would be expected to contain non-terminal peptides, though it is difficult to say what the 'chromophore' group in these peptides could have been.

The methods of investigation described in this paper should be generally applicable to all proteins and peptides that contain a free amino group. Besides giving information about the structure of the proteins, the method can probably be applied as a sensitive test for the identity and homogeneity of proteins. A simple experiment in which DNP-peptide bands are separated and the amino acids present in them identified can readily be carried out, and it is extremely unlikely that two different proteins would give the same result, probably less likely than that they would appear homogeneous by any of the known physicochemical methods of determining protein purity.

SUMMARY

1 The dinitrophenyl method for the identification of the terminal residues of proteins has been extended by the use of partial hydrolysis to the identification and estimation of terminal peptides.

2 The method was applied to insulin, and it was shown that both the terminal phenylalanyl residues are present in the form of the tetrapeptide sequence phenylalanyl valyl aspartyl glutamic acid, both the lysyl residues which are in the same chains are present in the sequence threonyl prolyl lysyl alanine and both the terminal glycyl residues are present in the pentapeptide sequence glycyl isoleucyl valyl glutamyl glutamic acid.

3 It is concluded that the insulin molecule is built up of two pairs of very similar, if not identical, polypeptide chains.

I wish to express my thanks to Prof. A. C. Chibnall, F.R.S., for his advice and encouragement.

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Regulation of Urinary Steroid Excretion

2 SPONTANEOUS CHANGES IN THE PATTERN OF DAILY EXCRETION IN MENTAL PATIENTS

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(Received 17 June 1949)

The daily excretion of 17 ketosteroids of a large series of mental patients has been estimated for several months—in some cases for more than a year. It was observed that in some cases the rate of daily excretion was fairly constant, but in others there were wide variations, far outside the possible error of determination. Some light has been thrown on the physiological mechanism responsible by a detailed long term study of steroid excretion in one patient with cyclical manic and depressive phases, and by the study of diurnal variations in 17 ketosteroid output in chronic schizophrenic patients before and after treatment.

EXPERIMENTAL

Estimation of steroid excretion. In all cases where the total neutral 17 ketosteroid fraction only was required, the simultaneous hydrolysis and extraction procedure of Callow, Callow, Emmens & Stroud (1939) was employed. These estimations were carried out at intervals over long periods, on 24–72 hr urine specimens of mental patients and normal subjects. In the case of the cyclic patient, however, the more detailed procedure for fractionation and estimation of the steroids, as previously described by us (Reiss, Hemphill, Gordon & Cook, 1949), was followed.

Diurnal variations were estimated by making use of the micromethod of Dreker, Pearson, Bartzak & McGavack

(1947) which requires only 10 ml urine, such estimations were carried out in duplicate, the margin of error being rather greater than in the macromethod. It was thus possible to estimate 17 ketosteroid output on urine collections taken every 3 hr throughout the day (7 a.m.–10 p.m.), ending with a 9 hr night collection (10 p.m.–7 a.m.).

RESULTS

Variations in 24 hr excretion. Fig 1 illustrates the excretion rates of total 17 ketosteroids in mg/24 hr for normal subjects, over periods of 6–14 months, and this diagram may be compared with the corresponding excretion rates for chronic schizophrenic patients, which are recorded in Fig 2. The excretion rates for normal subjects are relatively constant, varying at the most ± 1.5 mg/24 hr. Some of the patients also show excretion rates which approach this constant pattern (e.g. nos 7, 9, 10 and 12), but others show wide variations, which may amount to ± 6.0 mg/24 hr. These variations could not necessarily be correlated with the clinical picture. Further investigations designed to ascertain whether relationships exist between psychotic condition and constancy or inconstancy of ketosteroid excretion are at present being undertaken.

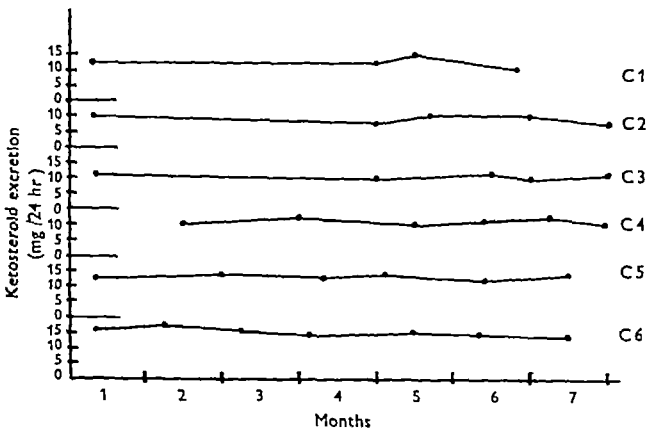


Fig 1 Variation of urinary steroid excretion in normal subjects (72 hr urine specimens)

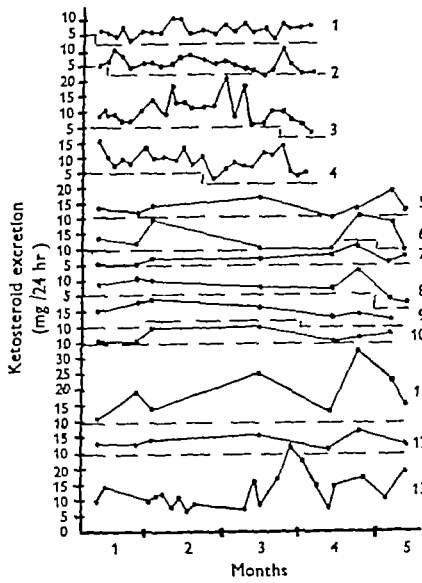


Fig 2 Variation of urinary steroid excretion in schizophrenic subjects (48 hr urine specimens)

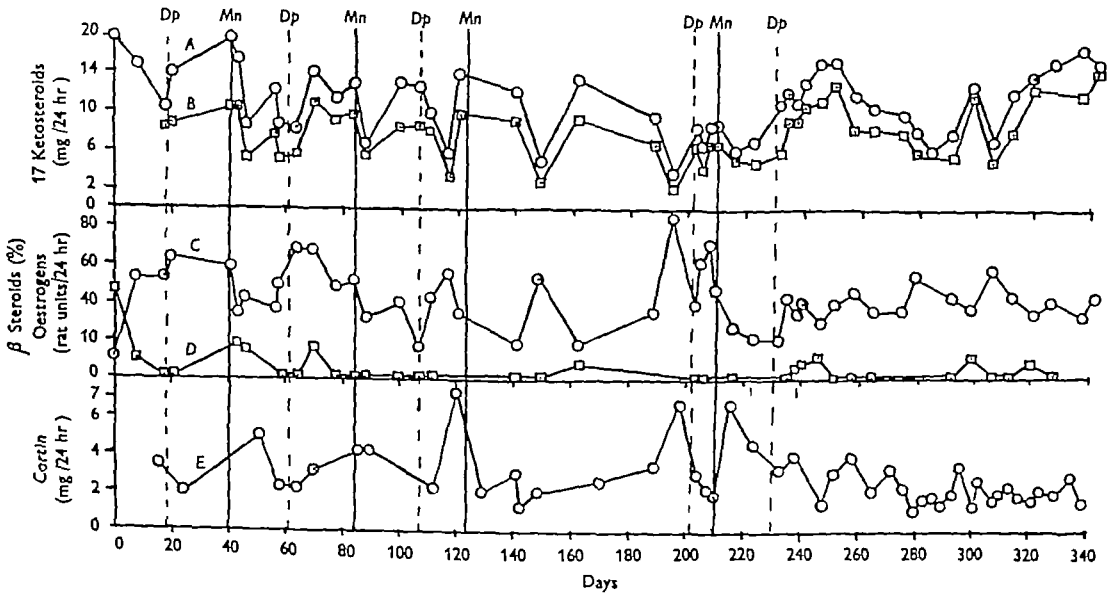


Fig 3 Variations of urinary steroid excretion in a case of manic depressive psychosis A, total 17 ketosteroids (mg/24 hr), B ketonic fraction of 17 ketosteroids (mg/24 hr) C, 3(β) hydroxy 17 ketosteroids (% of ketonic fraction), D, oestrogens (rat units/24 hr) E, cortin, mg/24 hr, Dp, times of change into depression Mn, times of change into mania

Fig 3 records the daily excretion rates of various steroid fractions for a 45 year old typical manic depressive psychotic patient, with relatively short phase cycles. Manic phases varied between 1 and 6 weeks, and depressive phases between 6 and 24 days, the transition from one state to another being

always rapid. The remission or interval of normality between manic and depressive phases was never more than a few days and merged gradually into the succeeding phase. Values are recorded for excretion of total 17 ketosteroids, ketonic fraction, percentage 3(β) hydroxy 17 ketosteroids in the ketonic

fraction, cortin and oestrone, estimated at intervals over a period of 1 year (dates of transition from phase to phase are included) The inconstancy of the excretion rates of all these fractions is particularly marked in this case, the possible magnitude of the variations being greater than in the other cases, for corresponding periods In as short a period as 10 days, changes of 100–300 % could occur, the cortin excretion, for instance, changed during each cycle by over 100 %, as also did that of 3(β)-hydroxy 17-ketosteroids During the course of the year, a total 17-ketosteroid excretion varying between 2 and 18 mg/24 hr was observed A closer examination of the variations in different fractions shows an inverse relationship between changes in β steroid and cortin

a tendency to maximal values during the hours shortly after waking, followed by a regular decline during the rest of the day This normal pattern was shown by only a few of the psychotic patients The results are illustrated in Fig 4 The following deviations from the normal may be noted (1) A greatly enhanced increase in the 'waking' output (7–10 a.m.) compared with the night output, sometimes amounting to 400 %, the corresponding figure for normal subjects being 60 % (Pincus & Hoagland, 1943) (2) Slight variations only during the day and night (3) Maximal excretion delayed until a later time during the day, instead of in the 7–10 a.m. period (4) A second peak in output occurring late in the day, in addition to the morning peak

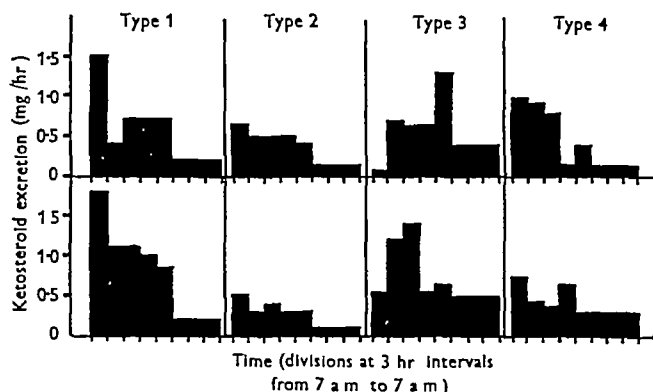


Fig 4 Diurnal variations of urinary 17 ketosteroid excretion

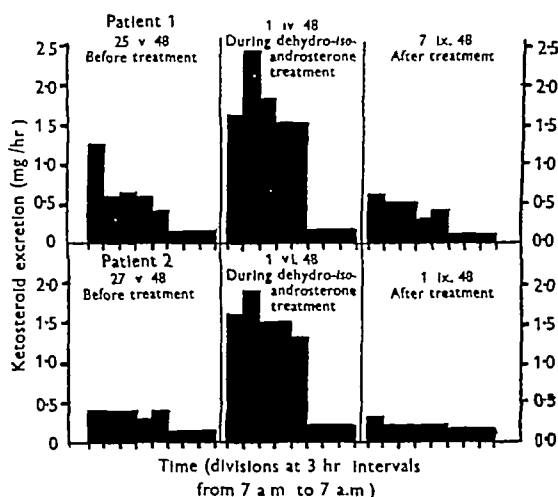


Fig 5 Influence of dehydroisoandrosterone on diurnal pattern of 17 ketosteroid excretion.

excretion—a similar inverse relationship between β steroid excretion and oestrone excretion was also sometimes noted The effect was most marked in the first regular cycles, where the onset of depression appeared to be accompanied by a tendency to excretion of a high proportion of β steroids and a low cortin excretion Cortin excretion was usually rising at the transition into mania, when at the same time the proportion of β steroids was falling The clear cut pattern of inverse relationship was followed by an irregular psychological period, during which time changes in ketosteroid excretion continued, unaccompanied by psychological changes From September onwards, a long period of depression was maintained, in which the average β steroid excretion was continuously high (over 20 %) and the cortin excretion comparatively low

Diurnal variations The variations in 17 ketosteroid excretion during the day and night were investigated in normal individuals by Pincus (1943), who reported minimal excretion during sleep and

Types 1 and 2 are both influenced by dehydroisoandrosterone treatment, as illustrated in Fig 5 Patient 2, a catatonic of 20 years' standing, underwent no change in mental state after the treatment, and it will be seen that his diurnal variation curve, which is of type 2, was not materially different after treatment had stopped, though it underwent considerable change during the course of the treatment Patient 1, on the other hand (a hallucinated and restless schizophrenic of 16 years' standing), whose diurnal pattern is of type 1, responded well to treatment, and was subsequently discharged from hospital, at the same time, the diurnal pattern was definitely changed after cessation of treatment, approximating more to the normal

DISCUSSION

A scrutiny of the ketosteroid excretion values of the psychotic patients, considered in relation to the known facts concerning their clinical condition,

showed that those whose excretion was constant over several months (nos 5, 7, 8, 9, 10 and 12) were older chronic schizophrenic patients (two of them catatonic) who had been in hospital for at least 10 years. The diurnal curves of this group, as far as they have been investigated, are of type 2, showing little variation throughout the 24 hr period. The other patients referred to in Fig 2 were restless and hallucinated. This group showed much sharper changes in the diurnal pattern, with a degree of variation several times greater than the normal maximum of 60% observed by Pincus & Hoagland (1943) and by us. In some, the excretion peak was delayed, while in others, more than one peak could be observed. It remains to be seen whether the differentiation of diurnal curves into the types 1, 2, 3 and 4 may become useful for diagnostic purposes. It is clear, however, that the resultant of the sharply changing diurnal curves illustrated by types 1, 3 and 4 is likely to produce the greater variations in daily output.

The diurnal variation of 17 ketosteroid excretion is an expression of the adaptation by the individual to the demands of his environment and daily life. Very little adrenal cortical activity is necessary during sleep, and the most essential demand in the 24 hr cycle is in the process of waking. It is, therefore, understandable that the excretion is at a maximum for normal subjects during the first few hours of the morning, and this adaptation mechanism is evidently disturbed in schizophrenic patients.

The daily excretion pattern of the manic depressive patient shows that different functions of the adrenal cortex need not change in a parallel manner, since the cortin like substances can show increases at the same time as the 3 (β) hydroxy 17 keto steroids are decreased, and vice versa. The antagonism between these two components was regularly observed, and changes in their values nearly always took place at times corresponding with changes in the mental state. The sudden change into depression took place while the β steroid excretion was rising, or at a maximum, at the same time as the cortin excretion was falling, the opposite changes were seen at the commencement of the maniacal phase. For one period during the summer months of the year the typical excretion pattern was unaccompanied by the usual mental changes. Towards the autumn of the year the patient went into a prolonged depression, during which, for more than 3 months, the β steroids remained high and the cortin excretion comparatively low.

It is important to consider the extent to which such changes in adrenal cortical activity are a primary cause, or a secondary consequence of the mental disturbance. In this connexion, the work of Allen, Broster, Vines, Patterson, Greenwood, Marrian & Butler (1939) may be taken into account.

These workers claimed that paranoid psychotics with adrenogenital virilism, who showed increased ketosteroid excretion, were improved mentally after unilateral adrenalectomy, when the ketosteroid level was also lowered. These are undoubtedly special cases, and while we do not suggest that the psychiatric condition was similar to those we have studied, the physiological mechanism for the production of some of the symptoms is probably the same. Mental changes after castration are generally known, and considerable disturbances in testicular function in schizophrenic patients were demonstrated by Hemphill, Reiss & Taylor (1944). Further, in the previous paper of this series (Reiss *et al* 1949) there was some indication that the 3 (β) hydroxy 17 ketosteroid, dehydroisoandrosterone, could influence the mental state. In the present study, the diurnal curve became more similar to the normal after administering dehydroisoandrosterone in the case of the patient whose mental condition improved, but not in the case of the patient who did not improve. It may be added that, in recent months, we have accumulated results of investigations, to be described later, on depressed patients showing abnormally high β steroid excretion, some of whom improved mentally after treatment with testosterone (which also brought about a decreased β steroid excretion). Hoagland (1944) reported improvement in work efficiency after administration of pregn 5-en-3-ol 20-one. It is also pertinent to remember that steroid hormones may influence the brain circulation (Reiss & Golla, 1940) and may also exert a narcotic like action (Selye, 1941).

The facts quoted above suggest that changes in hormone production may directly influence the mental state. It may be argued, however, that the response of the adrenal cortex to different demands is itself directed by vegetative brain centres, via the pituitary anterior lobe. It is perhaps permissible to visualize a cycle of events in which a disturbance of certain vegetative brain centres results in a dysfunction of the adrenal cortex—the latter in its turn influencing other brain parts and producing mental change. Further clarification of such a mechanism must await the accumulation of considerably more clinical and experimental evidence.

SUMMARY

1 Daily 17 ketosteroid excretion in the urine of mental patients, estimated at intervals for several months, showed either constant or widely varying values.

2 Different types of diurnal variation of 17-ketosteroid excretion have been recorded in mental patients.

3 A patient suffering from manic-depressive psychosis was found to excrete a high proportion of 3 (β) hydroxy 17 ketosteroids, accompanied by a

relatively low cortin value, during most of the depressive phases. The reverse changes were usually observed during manic phases.

4 The possible relationships between steroid hormone production and mental symptoms are discussed in the light of the above findings.

The authors wish to acknowledge their indebtedness to Messrs Organon Laboratories for supplies of dehydroisoandrosterone and to Miss B Binham and Messrs R J Beavan, D J Dell and W G Warren for technical assistance, also to Mr F Knight for valuable co operation in the collection of specimens.

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Optical Rotation of the Molybdate Complex of *Dextro-iso*Citric Acid

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(Received 24 June 1949)

Owing to the high optical rotatory power of the isocitrate citrate molybdate complex, milligram quantities of *dextro isocitric* acid can be determined polarimetrically (Martius, 1938, Jacobsohn, 1940, Martius & Leonhardt, 1943, Krebs & Eggleston, 1943). * The accuracy of the method depends, *inter alia*, on information concerning the specific rotation of this complex under the conditions of the test. Previous measurements, giving an $[\alpha]_D$ value of -780° , could not be regarded as highly accurate as the isocitric acid used for the measurements was not pure (Krebs & Eggleston, 1943). Dr H B Vickery has recently given us a sample of pure dimethyl isocitrate lactone prepared from the isocitric acid of *Bryophyllum* leaves (Pucher, Abrahams & Vickery, 1948). This enabled us to make more precise measurements of the rotation of the complex in the conditions used for the quantitative determination of isocitric acid.

EXPERIMENTAL

To prepare a stock solution of *dextro isocitric* acid 202 mg (1 mmol) of the dimethyl ester lactone were dissolved in 20 ml 0.25N NaOH and kept at 40° for 30 min. This treat-

ment was found to be sufficient for the quantitative conversion of the substance into free isocitric acid. The stock solution was diluted as required.

The standard test conditions were as follows (see Krebs & Eggleston, 1943). To 10 ml of the solution to be tested were added 1.25 ml glacial acetic acid, 2.5 ml. N sodium citrate and 11.25 ml. 29% (w/v) ammonium molybdate. (Citrate was included because it increases the rotation of the complex (Krebs & Eggleston, 1943, Martius & Leonhardt, 1943).) After mixing and filtration the solution was transferred to a 2 dm water jacketed Hilger polarimeter tube. Rotation readings were taken at different temperatures. The light source was a sodium lamp.

The results of the measurements at different temperatures are given in Table 1. The values for $[\alpha]_D$ are calculated for anhydrous isocitric acid, not for the molybdate complex (the structure of which is unknown). The concentration of isocitric acid in the test solution, as given in the top line of Table 1, refers to the concentration of the solution in the polarimeter tube, i.e. after the addition of the various reagents.

The data show that $[\alpha]_D$ decreases with rising temperature and also, slightly, with rising concentration of isocitric acid. It is therefore important in polarimetric determinations to control the temperature and to select an $[\alpha]_D$ figure for the calculation which is valid for the concentration range under test.

Further data for $[\alpha]_D^{20}$ at ten different concentrations of isocitric acid are shown in Table 2. It should be noted that the absolute error of the measurements is constant (about $\pm 0.02^\circ$ for α), and that the percentage error in α is therefore greater the smaller the value of the reading.

* The term '*dextro-isocitric* acid' is used in accordance with the conventions laid down in *Principles of Abstracting* (Bureau of Abstracts, 1948). The substance has previously been called '*d-isocitric* acid' (Pucher, Abrahams & Vickery, 1948) and '() isocitric acid' (Krebs & Eggleston, 1944).

Table 1 *Rotation of the dextro isocitrate citrate molybdate complex*

(For test conditions see text, α_D is the rotation in degrees observed in a 2 dm tube, $[\alpha]_D$ the specific rotation calculated for isocitric acid)

Concentration of isocitric acid (mg /ml.)	Temp (°)	0 48		0 96		1 92		3 84	
		α_D	$[\alpha]_D$	α_D	$[\alpha]_D$	α_D	$[\alpha]_D$	α_D	$[\alpha]_D$
	10	-0 80°	-834°	-1 56°	-812°	-2 98°	-776°	-5 39°	-701°
	15	-0 77°	-802°	-1 50°	-781°	-2 82°	-735°	-5 18°	-675°
	20	-0 75°	-781°	-1 43°	-745°	-2 66°	-693°	-4 96°	-645°
	25	-0 73°	-760°	-1 38°	-719°	-2 51°	-654°	-4 75°	-618°
	30	-0 70°	-729°	-1 32°	-688°	-2 35°	-612°	-4 54°	-591°
	35	-0 68°	-709°	-1 26°	-657°	-2 19°	-570°	-4 33°	-564°

Table 2 *Effect of the concentration of dextro iso citric acid on $[\alpha]_D$ in solutions containing the isocitrate citrate molybdate complex*

(Standard conditions. 2 dm. tubes 20° $[\alpha]_D$ calculated for isocitric acid.)

isoCitric acid concentration (mg /ml solution tested)	α_D observed	$[\alpha]_D$
0 240	-0 40°	-834°
0 480	-0 75°	-781°
0 576	-0 89°	-772°
0 672	-1 02°	-758°
0 768	-1 15°	-750°
0 864	-1 29°	-747°
0 960	-1 43°	-745°
1 44	-2 09°	-726°
1 92	-2 66°	-693°
3 84	-4 96°	-645°

In the previous experiments (Krebs & Eggleston, 1943) the rotation measured was about -1 80° (2 dm tubes) at

approximately 18° According to Table 1 $[\alpha]_D^{18}$ for these conditions is -735°, whilst it was previously assumed to be -780° A small correction of the previously calculated iso citric acid values is therefore required The equilibrium mixture of citrate, isocitrate and cis aconitate had been taken to contain 6 2% isocitrate (at 38°) The recalculation gives a value of 6 6% isocitrate

SUMMARY

The optical rotatory powers of solutions containing the isocitrate-citrate molybdate complex have been measured under varying conditions, in order to obtain reference values for the polarimetric determination of dextro isocitric acid

We are indebted to Dr H B Vickery, Connecticut Agricultural Experiment Station, New Haven, Conn , for a sample of dextro isocitric acid prepared by the late Dr G H. Pucher

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The Use of Buffered Columns in the Chromatographic Separation of 2 4-Dinitrophenyl Amino-acids

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(Received 27 June 1949)

During an investigation of the action of papain on wool keratin, the elegant method of Sanger (Sanger, 1945, Porter & Sanger, 1948) was used to determine the end groups of the peptides liberated by the enzyme In this method, the terminal amino groups of proteins or polypeptides are condensed with 1 2 4 fluorodinitrobenzene and the amino acids originally carrying free amino groups are isolated

chromatographically as their coloured N-2 4 dinitrophenyl (DNP) derivatives after acid hydrolysis

It was observed at an early stage that silica gels which had been successfully employed for the separation of N acetyl amino acids were of little use for the separation of DNP amino acids Using chloroform as the solvent, for example, practically

all the DNP amino acids ran together as one fast band. Consden, Gordon, Martin & Syngé (1947), who had a similar experience, were of the opinion that the action of Sanger's gels must be attributed to the occurrence of strong adsorption effects in addition to the effect of distribution between the phases. Sanger (1945) originally found the *R* values (Martin & Syngé, 1941) of DNP amino acids to vary considerably with the batch of silica, and considered that they were adsorbed on the silica to a certain extent. Phillips & Stephen (1948) have also commented on the variation between different batches of gel.

The successful use of buffered columns of silica gel in the separation of the penicillins by partition chromatography (Boon, 1948) suggested that the expedient might be useful in the separation of DNP amino acids.

METHODS

Preparation of silica gel. The general procedure of Gordon, Martin & Syngé (1943, 1944) and Tristram (1946) for the preparation of silica gel was followed. After precipitation of the gel, a considerable excess of HCl was added to the mixture, which was then allowed to stand for 3 hr. After filtration and washing, the gel was allowed to stand for 3 days in distilled water, and was again washed and dried. The gel prepared in this way was a 'non adsorbent' gel.

Preparation of DNP amino acids. The DNP derivatives of glycine, DL-alanine, DL-valine, DL-isoleucine, DL-leucine, L-proline, DL-phenylalanine, DL-methionine, DL-aspartic acid, DL-threonine, DL-serine and L-tryptophan were prepared by the method of Abderhalden & Blumberg (1910). Bis DNP L-lysine was prepared by the same method and ϵ DNP L-lysine according to Porter & Sanger (1948). All these derivatives were recrystallized from appropriate solvents before use and had melting points in good agreement with those in the literature. As reported by Sanger (1945), DNP L-glutamic acid did not crystallize. DNP DL-glutamic acid was prepared by the method of Abderhalden & Blumberg (1910). The cooled reaction mixture, after concentration *in vacuo* to remove the greater part of the ethanol, was diluted and extracted with ether. The heavy oil which precipitated on acidification solidified after standing overnight. It was recrystallized twice from ethyl acetate-cyclohexane and had m.p. 172° (Found C, 42.3, H, 3.6, N, 13.6, $C_{11}H_{11}O_8N_3$ requires C, 42.2, H, 3.5, N 13.4%). All the above compounds were found to give single bands on appropriate silica gel columns. The reaction between L-tyrosine and 1,2,4-chlorodinitrobenzene gave a substance forming a single band on a buffered $CHCl_3$ column. This was presumably bis DNP L-tyrosine, since the compound formed by the action of 1,2,4-fluorodinitrobenzene on L-tyrosine had a similar rate (Sanger, 1945).

Preparation of columns. In general the customary technique of partition chromatography was employed, 66% by weight of a suitable buffer solution being added to the dry gel before pouring the column. The buffers employed are conveniently distinguished as A, B, F and G. Buffer A was a 2M solution of NaH_2PO_4 . Buffers F, B and G were Sørensen's phosphate buffers (Britton, 1942, Table 76) of pH 6.47, 6.98 and 6.24 respectively. The concentrations of salts in these buffers were increased so that the molar con-

centrations of H_2PO_4 in them were respectively 0.25, 0.5 and 0.25. The pH of buffers A, B, F and G, as measured by the glass electrode, were 3.72, 6.61, 6.16 and 5.95 respectively.

It was observed during the course of this work that DNP amino acids tended to decompose if exposed to sunlight on the column. Solutions of DNP amino acids in solvents such as $CHCl_3$ also tended to decompose when exposed for long periods to light, particularly sunlight. The solutions then gave rise to additional bands on the chromatogram. The practice was therefore adopted of evaporating the solvent as soon as the band under consideration had run out of the column, and where necessary refractionation was carried out without undue delay. Ethanol solutions of DNP amino acids left for some weeks in a cupboard were found to give rise to fast yellow bands on columns in addition to the bands due to the DNP amino acid.

RESULTS

Using buffered columns of silica gel and water-saturated chloroform as solvent, mixtures of DNP amino acids can readily be separated into distinct bands. By varying the pH of the buffer, and hence the pH of the aqueous phase, the band rate of any particular DNP amino acid can be varied within wide limits. As the pH of the aqueous phase of the column is increased, the band rate of any particular DNP amino acid decreases. The band rates can easily be made slow enough to separate DNP amino acids by increasing the pH of the buffer.

Table 1 gives the band rates of a number of DNP amino acids on columns buffered to different pH values in chloroform and *n*-butanol-chloroform mixtures. The behaviour of DNP-L-hydroxyproline was not studied, since it was known to be absent from the mixtures it was desired to investigate. Over the pH range studied, the variation of band rate with pH of the column is most marked in the case of the slower moving DNP amino acids. Using a single solvent such as chloroform in conjunction with different buffered columns, it is possible to effect separation of DNP amino acids which cannot be accomplished by the use of a single unbuffered column with the same solvent. The use of buffered columns largely eliminates the variations in behaviour between batches of silica gel which previous workers have commented on. A number of solvent systems have been found useful when employed with buffered columns. These systems and the type of separation they will accomplish are briefly described below. A description is also given of the method of separation of DNP amino acid mixtures based on these solvents and buffers.

Chloroform and butanol-chloroform columns

Buffer A $CHCl_3$ columns are useful for the separation of DNP monoaminomonocarboxylic acids from DNP glutamic acid, DNP aspartic acid, DNP threonine and DNP serine. These columns will also separate DNP glutamic acid from

Table 1 *Band rates (R values) of DNP amino-acids in chloroform and butanol-chloroform*

DNP amino acid	R values			
	CHCl ₃ , buffer A	CHCl ₃ , buffer B	3% butanol CHCl ₃ , buffer G	17% butanol CHCl ₃ , buffer B
DNP leucine	Fast	0.7	—	—
DNP valine	Fast	0.5	—	—
DNP phenylalanine	Fast	0.7	—	—
DNP methionine	Fast	0.5	—	—
DNP proline	Fast	0.3	—	—
DNP alanine	Fast	0.2	—	0.5
DNP tryptophan	0.6	0.4	Fast	—
DNP glycine	0.9	0.03	0.5	0.2
Bis-DNP lysine	0.7	0.06	Fast	Fast
Bis DNP tyrosine	Fast	0.07	0.9	Fast
DNP threonine	0.3	Slow	0.18	0.15
DNP serine	0.11	Slow	0.08	0.07
DNP glutamic acid	0.17	Slow	0.2	Slow
DNP aspartic acid	0.06	Slow	Slow	Slow

DNP aspartic acid and DNP threonine from DNP serine. Buffer B CHCl₃ columns will separate DNP alanine from DNP glycine and DNP valine from DNP alanine. The band rates of DNP amino acids in CHCl₃ on columns buffered to a more alkaline pH (Sørensen's pH 8 phosphate buffer, 0.25M (Britton, 1942, Table 76) pH measured by glass electrode 7.84) were similar to those on buffer B CHCl₃ columns. Use of these columns did not separate those DNP amino acids not separated on buffer B CHCl₃ columns. The rates of DNP amino acids on buffer B CHCl₃ columns are roughly similar to those found by Sanger (1945) on CHCl₃ columns. Buffer B, however, probably gives less acid columns than does Sanger's silica gel.

In general, maximum separation is achieved with the least acid columns, although the band rates may then be inconveniently slow. Convenient separations of the slower moving DNP amino acids can often be achieved by employing butanol CHCl₃ instead of chloroform as the solvent. A mixture of DNP-glycine and bis DNP lysine or bis DNP tyrosine does not separate readily on a buffer B CHCl₃ column since all three move slowly under these conditions. On a buffer B 3% butanol-CHCl₃ column, however, the two latter DNP amino-acids move faster than DNP glycine and are readily separated from it.

Similarly, a mixture of DNP threonine, DNP serine and DNP aspartic acid can be separated into three bands, emerging from the column in the above order, on buffer G 3% butanol CHCl₃ columns or on buffer B 17% butanol CHCl₃ columns. With a change in the solvent and buffer in this way, the order in which the DNP amino acids emerge from the column may be altered. Thus on buffer A-CHCl₃ columns DNP glutamic acid emerges ahead of DNP serine. On more alkaline columns, with butanol CHCl₃ as the mobile phase (on buffer B 17% butanol-CHCl₃ columns), the R value of DNP serine is greater than that of DNP glutamic acid. This behaviour may depend on the fact that the ionization of DNP glutamic acid can involve two carboxyl groups, as opposed to one in the case of DNP monoamino monocarboxylic acids. On the gels used by Sanger with 1% butanol CHCl₃ as the mobile phase, DNP threonine and DNP serine have faster rates than DNP glutamic acid and DNP aspartic acid (Sanger, 1945, Porter & Sanger 1948).

Propanol cyclohexane columns

Propanol cyclohexane (5%) used with buffered columns is a very useful solvent for the separation of DNP monoamino monocarboxylic acids. The rates of the DNP amino acids on these columns are also dependent on the pH of the aqueous phase. Table 2

Table 2 *Band rates (R values) of DNP amino-acids in 5% propanol cyclohexane*

DNP amino acid	R values (5% propanol-cyclohexane)		
	Buffer A	Buffer B	Buffer F
DNP leucine	—	0.4	0.5
DNP valine	Fast	0.15	0.4
DNP phenylalanine	—	0.2	0.3
DNP methionine	—	0.06	0.08
DNP proline	—	—	0.06
DNP alanine	Fast	0.04	0.10
DNP tryptophan	—	—	0.11
DNP glycine	—	0.02	0.02

gives the rates of DNP amino acids in this solvent. From the band rates quoted by Sanger (1945) for propanol cyclohexane columns it is evident that buffer F gives less acid columns than did Sanger's gels with this solvent. It has been found that buffer F columns are suitable for the separation of the majority of the DNP amino acids of this group. Buffer B columns may be used for the separation of the longer chain DNP amino acids. The shorter chain DNP monoamino acids move rather slowly on buffer F 5% propanol cyclohexane columns, but buffer G 10% propanol cyclohexane columns gives rates suitable for convenient separation.

On buffer F-5% propanol-cyclohexane columns DNP valine can be separated from DNP alanine, and DNP alanine from DNP glycine. DNP isoleucine and DNP valine can be readily separated on a buffer B 5% propanol cyclohexane columns. A mixture of DNP isoleucine and DNP phenylalanine can also be separated on the latter

column, as can a mixture of DNP valine and DNP methionine. A mixture of DNP isoleucine, DNP valine, DNP alanine and DNP glycine separates into four bands on this column, but the rates of DNP alanine and DNP glycine are rather slow.

These columns thus generally affect the separations that can be achieved on ethanol-ligroin columns made according to Sanger (1945). It is noteworthy that both these solvent systems contain alcohols. The type of separation which can be brought about by a given solvent system may therefore be correlated with the chemical character of the solvent. Martin (1948) has previously pointed out the relationship existing between the chemical constitution of the solvent and the extent of separation of amino acids and their derivatives in partition chromatography.

Ether columns

Buffer *A*-ether columns can be used to free ether extracts of hydrolysates of DNP-proteins and polypeptides from HCl (cf Sanger, 1945) as all the DNP amino acids travel fast on these columns. On one occasion when an ether extract of DNP amino acids from the hydrolysate of a protein treated with 1,2,4-fluorodinitrobenzene was passed through a buffer *G* ether column to remove HCl, yellow bands, presumably DNP amino acids, were observed passing down the column. Investigation showed that the DNP amino acids moved on buffer *B* ether columns with characteristic rates, which are given in Table 3. These columns are useful for separating the slower moving DNP amino acids from each other.

Table 3 *Band rates (R values) of DNP amino-acids in ether and 33 % ether ligroin*

DNP amino acid	Ether, buffer <i>B</i>	33 % ether ligroin, buffer <i>B</i>
DNP leucine	Fast	0.5
DNP valine	Fast	0.2
DNP phenylalanine	Fast	0.1
DNP methionine	1.0	0.1
DNP proline	0.5	0.04
DNP alanine	0.5	0.08
DNP tryptophan	Fast	0.09
DNP glycine	0.2	0.04
Bis DNP lysine	0.6	(Sparingly soluble)
Bis DNP tyrosine	0.9	(Sparingly soluble)
DNP threonine	0.14	—
DNP serine	0.10	—
DNP glutamic acid	0.04	—
DNP aspartic acid	0.01	—

A mixture of DNP threonine, DNP serine and DNP aspartic acid separates into three bands, whilst DNP threonine or DNP serine can be separated from DNP glutamic acid. DNP glutamic acid separates from DNP aspartic acid, but the band rates are rather slow for convenient separation. DNP amino acids which do not separate readily on buffer *B* CHCl₃ columns will often do so on buffer *B* ether columns. Thus bis DNP tyrosine or bis DNP lysine will separate from DNP glycine. The faster

moving DNP amino acids also separate on buffer *B* ether columns. The band rates are somewhat high, but if more alkaline buffers are employed the bands 'tail' into each other. A mixture of DNP valine, DNP alanine and DNP glycine separates into three bands, and DNP alanine can be separated from DNP methionine or DNP tryptophan.

Ether ligroin columns

The longer chain DNP monoamino acids have characteristic rates on buffer *B* 33 % ether ligroin columns (Table 3). These acids move fast on buffer *B*-ether columns. A mixture of DNP-valine, DNP-alanine and DNP glycine separates into three bands on a buffer *B*-33 % ether-ligroin column. An extensive investigation has not yet been made, but these columns seem to effect the same separations as buffer *B* CHCl₃ columns.

Columns employing organic solvents as the stationary phase

When 'non adsorbent' gels were employed it was found that certain of Sanger's columns, which use liquids such as aqueous ethanol or aqueous acetone as the stationary phase, did not separate the appropriate mixtures of DNP amino acids clearly. Thus with the gels prepared by the present author a mixture of DNP valine and DNP-phenylalanine was not clearly separated on acetone-cyclohexane columns prepared according to Sanger (1945). When one volume of acetone was mixed with one volume of buffer *G* instead of water and the column then prepared as before, the expected separation was readily obtained.

The separation of DNP alanine from DNP-proline was also difficult on glycol benzene columns using 'non adsorbent' gels. Using the same solvent system both DNP proline and DNP alanine moved extremely slowly on a gel which had been prepared by precipitating sodium silicate with acid and washing with buffer (buffer *G*) before drying.

Band rates of acid soluble DNP amino acids

The band rates of the acid soluble DNP amino acids were also found to vary with the pH of the aqueous phase of the column. No systematic investigation of this variation was made, but it was found that columns buffered with buffer *A* gave the desired separations with 66 % methyl ethyl ketone ether and 30 % butanol chloroform as the moving phase. These are the solvent systems used by Sanger for these separations.

Separation of mixtures of DNP amino acids

From the present experiments, and those of Sanger, it is evident that a mixture of DNP amino acids can be separated into its constituents in a number of different ways, as can a mixture of *N*-acetyl amino acids (Tristram, 1946). The number of

fractionations necessary to effect a complete separation obviously depends on the number of DNP amino acids present in the mixture being studied. Buffered columns have been successfully employed by the present author in separating DNP amino acid mixtures, and the scheme employed differs from that elaborated by Porter & Sanger (1948). The initial fractionation is generally carried out on buffer *B* ether columns, although buffer *G* 3% butanol chloroform columns were employed in the earlier stages of this work. On buffer *B*-ether columns the longer chain DNP monoamino acids move rapidly down the column followed by DNP glycine, DNP-threonine and DNP serine, which can be separated and collected. DNP glutamic and DNP aspartic acids form a slow-moving band at the top of the column which splits into its constituents on continued development. Elution of these two bands separately with ether usually required too long a time and it is convenient to elute them together with ether containing a few drops of glacial acetic acid to make the column acid. These DNP amino acids then run rapidly through the column and, after taking the solution to dryness, can be fractionated on a buffer *A* chloroform column. The fast moving first band obtained on buffer *B*-ether columns contains the DNP monoaminomonocarboxylic acids. After taking to dryness, it is refractionated on a buffer *B* chloroform column. The bands from this fractionation are then further refractionated on buffer *G* 10% propanol cyclohexane columns for the slower-moving DNP amino acids and on buffer *B* 5% propanol cyclohexane columns for the faster moving DNP amino acids.

Sanger (1945) observed that partition chromatography on paper was not successful in separating mixtures of DNP amino acids, owing to 'tailing' of the spots. Phillips & Stephen (1948) claimed some success, particularly with the slower moving DNP amino acids, using a two dimensional technique. Experiments by the present author with a wide variety of solvents led to similar conclusions to those of Sanger (1945), only a partial separation of the different DNP amino acids being achieved. It is probable that adsorption of the DNP amino acids on the paper occurs and this effect is superimposed on the partition effects. There is some evidence that adsorption does occur, since when DNP amino acids were run in distilled water under conditions similar to those of capillary analysis (Rheinboldt, 1925) fairly well defined spots were formed at some distance from the solvent front. The difference in rate between the different DNP amino acids was not sufficient to effect separations.

In a few experiments employing partition chromatography, paper previously soaked in buffer solution and then dried was used, but no marked increase in the effectiveness of the separation was achieved.

Alumina treated paper (Datta & Overell, 1949) similarly showed no marked improvement in separation over ordinary paper in a limited number of experiments. Columns of powdered cellulose saturated with buffer solutions effected some degree of separation of DNP amino acids, but were not as successful as silica gel columns.

DISCUSSION

Sanger (1945) and Consden *et al* (1947) considered that the DNP amino acids are adsorbed to a certain extent on the silica gel. The theory of the partition chromatogram, as initially worked out by Martin & Synge (1941), assumes that the partition coefficient of the solute remains constant with varying concentration, but, as they pointed out, this is seldom the case. If appreciable ionization of the solute, e.g. a DNP amino acid or *N* acetyl amino acid, occurs in the aqueous phase the partition coefficient will vary appreciably with the concentration. As a result the forward boundary of the chromatogram band will become sharper and the back more diffuse, a result commonly observed in ordinary 'adsorption' chromatography where the adsorption follows a Langmuir type isotherm (Tiselius, 1947, Cannan, 1946). Tiselius emphasized that there was hardly any difference in principle between ordinary and partition chromatography. By making the pH of the aqueous phase of the column sufficiently high, the ionization of the DNP amino acid in the aqueous phase becomes appreciable and the band formed on the chromatogram will be of the adsorption type. The adsorption effects noted by Sanger (1945) and Consden *et al* (1947) are thus probably due to small amounts of alkali retained by the gel during its preparation. Porter & Sanger (1948) found that adsorbent gels giving low *R* values produced maximum resolution for any given solvent, but gave 'tailing' bands. The present author believes that variations in behaviour between different batches of gel using unbuffered columns are due in large part to variations in the pH of the aqueous phase in contact with the gel. Using a given buffered column, no significant variation has been observed in the rate of a particular DNP amino acid when different batches of gel are employed. This indicates that variations between different batches of gel, e.g. in the amount of acid or alkali retained, do not cause significant variations in the pH of the aqueous phase of a buffered column. Nevertheless, the band rate of any particular DNP amino acid varies with the amount of material placed on the column and with the distance the band has travelled down the column. It is possible that some of the variations between batches of gel observed in the separation of *N* acetyl amino acids (Tristram, 1946, Martin, 1948) might be eliminated by the use of buffered columns.

The present buffers and solvent systems may not necessarily be the best or the most convenient for separating mixtures of DNP amino acids, and other schemes of separation using buffered columns could almost certainly be devised. The primary object of the present work was not centred in devising the best analytical method, but in eliminating some of the difficulties encountered using unbuffered columns. The application of these methods will be described in a forthcoming paper.

SUMMARY

The use of buffered silica gel columns in the partition chromatographic separation of 2,4-dinitrophenyl amino acids is described.

I am indebted to the Director and Council of the Wool Industries Research Association for permission to publish this paper and to Mr G. R. Lee for assistance with the experimental work.

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The Mechanism of the Action of Notatin

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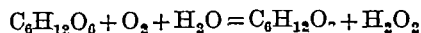
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(Received 15 July 1949)

Notatin is an enzyme discovered by Muller (1928) in *Aspergillus niger* and in *Penicillium glaucum*, and named by him glucose oxidase. Muller showed that this enzyme catalyses the oxidation of glucose by molecular oxygen to an acidic product which he identified as gluconic acid. Subsequently it was found by Franke & Lorenz (1937), using a more highly purified preparation, that hydrogen peroxide was formed in this reaction; these authors also suggested that the enzyme was a flavoprotein. A more complete purification was achieved by Coulthard, Michaels, Short, Sykes, Skrimshire, Standfast, Burkinshaw & Raistrick (1942, 1945), who named the enzyme notatin. Independently, Van Bruggen, Reithel, Cain, Katzman, Doisy, Mur, Roberts, Gaby, Homan & Jones (1943) also purified the same enzyme, designated by these authors penicillin B. The experiments of the American workers suggested that the prosthetic group was the alloxazine adenine dinucleotide. This was definitely proved by Keilin & Hartree (1946, 1948*a, b*), who examined the physical

and chemical properties of the enzyme in great detail and also determined its specificity range very thoroughly.

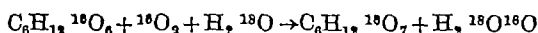
The overall reaction catalysed by notatin is generally represented as follows:



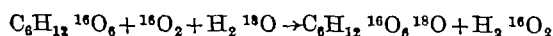
This equation does not make clear which is the essential, enzyme catalysed step in the reaction and, indeed, there has been some discussion as to whether notatin is a dehydrogenase (Franke & Lorenz, 1937; Franke & Deffner, 1939), or an oxidase (Müller, 1936). The latter hypothesis assumes that the enzyme 'activates' molecular oxygen, one of the oxygen atoms combining with the aldehyde form of glucose, whilst the other reacts with water to form hydrogen peroxide. However, the fact that notatin contains alloxazine-adenine dinucleotide suggests that the enzyme is a dehydrogenase. According to this second concept notatin catalyses the transfer of two hydrogen atoms from glucose or from the

hydrated aldehyde form of glucose (Franke & Lorenz, 1937) to molecular oxygen and the latter acts entirely as a hydrogen acceptor

The present authors (see Bentley, 1948) have found that the oxygen obtained from the decomposition of hydrogen peroxide, by catalase, in water enriched with respect to ^{18}O , contains the normal abundance of ^{18}O . Under these experimental conditions, therefore, there is no exchange of oxygen atoms between hydrogen peroxide and water during the decomposition of the hydrogen peroxide. It thus appeared possible to establish definitely the mechanism of the action of notatin by carrying out the glucose oxidation in water enriched with ^{18}O , and subsequently decomposing the hydrogen peroxide with catalase. If the notatin acts as an oxidase, the oxygen evolved on decomposition of the peroxide should contain half the isotope excess of the water used as a solvent



If, on the other hand, gaseous oxygen is a hydrogen acceptor in this reaction, the hydrogen peroxide will not contain any excess ^{18}O



EXPERIMENTAL

Materials

Notatin This was a highly purified preparation supplied to us by Prof. D. Keilin.

Catalase Preparations were made from horse liver and horse erythrocytes by the methods of Sumner, Dounce & Frampton (1940) and of Bonnichsen (1947), respectively. The haematin content of the catalase solutions was determined according to Keilin & Hartree (1936). The solutions used were between 10^{-7} and 10^{-8}M .

Glucose. In most of the isotope experiments a commercial sample of glucose hydrate was used which consisted almost entirely of the α form. Pure anhydrous α glucose was prepared according to Hudson & Dale (1917), it had $[\alpha]_D^{15} = +101.5^\circ$ (5 min.). β Glucose was prepared by the pyridine method of Behrens (1907) and recrystallized from aqueous ethanol as described by Hudson & Dale (1917), it had $[\alpha]_D^{18} = +20.8^\circ$ (6 min.).

Isotope experiments in H_2^{18}O

These experiments were carried out in a flask (25 ml. capacity) attached through pressure tubing to a 10 ml. gas burette. The flask could be shaken mechanically.

Glucose monohydrate was dissolved in H_2^{18}O and the notatin added in a length of melting point tubing so that initially it did not dissolve. The apparatus was swept out with normal O_2 and then closed with the burette full of O_2 . The flask was agitated so that the notatin dissolved and then shaken mechanically at room temperature, the O_2 uptake being followed in the burette. When there was no further change, the contents of the flask were transferred to one leg of the gas sample tube described by Sprinson & Rittenberg (1948). * The other leg contained a few drops of

a catalase solution. The solutions were frozen in an ethanol CO_2 bath and the tube evacuated to 10^{-3} mm. The tube was then closed by turning the cap, and the solutions allowed to thaw and degas. The contents were again frozen and the evacuation was completed. After thawing, the solutions were mixed to decompose the H_2O_2 and the liberated O_2 admitted to the sample system of the mass spectrometer in the usual way. Water samples were analysed by equilibration with CO_2 in the presence of carbonic anhydrase (Cohn & Urey, 1938; Bentley, 1948).

Possible exchange of oxygen in the decomposition of H_2O_2 by catalase. In a large number of control experiments H_2O_2 at various concentrations was decomposed, *in vacuo*, by catalase in the presence of H_2^{18}O (1.39 atom % excess ^{18}O). The evolved O_2 was analysed in the mass spectrometer, but was never found to contain a significant excess of ^{18}O . In view of the relatively low degree of enrichment of ^{18}O in the water used, it cannot be concluded that no exchange occurs in the catalytic decomposition of H_2O_2 . But it follows that if such an exchange occurs, it must be relatively small and would not affect the interpretation of the experiments reported in this paper. In further control experiments it was shown that there was no isotope exchange between O_2 and H_2^{18}O on shaking in the presence of notatin.

Experiments in $^{18}\text{O}_2$

Glucose monohydrate dissolved in normal water was placed in one leg of the gas sample tube of Sprinson & Rittenberg (1948) and the dry notatin in the other leg. The solutions were degassed and the tube evacuated as described above. The tube and contents were kept in an ethanol CO_2 bath and connected to the anodic part of an apparatus (Bentley, 1949) in which H_2^{18}O was electrolysed. When the required quantity of $^{18}\text{O}_2$ had been liberated, the tube was closed. The contents were allowed to thaw and the notatin dissolved by mixing. The tube was shaken at room temperature until the reaction was judged complete (3–4 hr.). The contents were frozen and a sample of the $^{18}\text{O}_2$ remaining in the tube admitted to the mass spectrometer for isotope analysis. The solution was washed into one leg of the tube and the H_2O_2 decomposed with catalase as described above. The O_2 obtained was analysed in the mass spectrometer.

Potentiometric and polarimetric experiments

Another series of experiments was carried out with the purpose of identifying the primary product of the action of notatin on glucose. In these experiments α or β glucose (anhydrous) was dissolved in water (with or without buffer), 0.2% (w/v) notatin and about $1 \times 10^{-3}\text{M}$ catalase were then added and the solution made up to volume with water. The solution was then transferred to a conical flask (100 ml.) fitted with inlet and outlet tubes. O_2 was passed in a fairly rapid stream through the inlet tube whilst the flask was shaken mechanically. The temperature in all experiments was $30 \pm 0.2^\circ$, unless otherwise stated.

Polarimetric measurements were made with a 2 dm. tube at $19\text{--}20^\circ$. The pH was measured with a glass electrode using a Cambridge Instrument Co. portable pH meter.

* One of us (R. Bentley) is very much indebted to Dr. Rittenberg for a description of this tube in advance of publication.

Enzymic oxidation was followed either by estimating the remaining glucose by titration with hypoiodite (Willstätter & Schudel, 1918) or by titrating alkalimetrically the total gluconic acid formed

Total gluconic acid As will be shown later, a lactone of gluconic acid is the first product of the enzymic reaction. It was therefore necessary, in order to estimate the combined and free gluconic acid and lactone, to boil the solution after each addition of alkali. The latter was added until the pink colour of phenolphthalein persisted for at least 1 min. The blank values obtained by titrating samples withdrawn at zero time were generally very small unless appreciable amounts of buffer were used. The values obtained by the two methods agreed generally within 3%. However, the alkalimetric method was found to be more reliable and figures for oxidation are based only on the total gluconic acid values, unless otherwise stated.

Free gluconic acid This was estimated by titrating a sample with 0.05N NH_3 at 1–3° to a pH of about 6.0–6.5, using B.D.H. Universal Indicator. The results obtained by this method were only accurate within about 5–10%, since hydrolysis of the lactone during the titration could not be completely avoided.

RESULTS

Isotope experiments

Experiments with H_2^{18}O Table 1 shows that there is no detectable isotope excess in the hydrogen peroxide produced in the notatin reaction, if ordinary oxygen and isotopically enriched water are used. The figures for oxygen uptake indicate that only about 80–90% of the total glucose has been oxidized. This is caused by the accumulation of hydrogen peroxide owing to the absence of catalase in the first phase of the experiment, and also by the absence of buffer.

Table 1 Atom % excess ^{18}O in hydrogen peroxide formed on oxidation of glucose by notatin with $^{18}\text{O}_2$ in H_2^{18}O

(Experimental error of isotope analysis for ^{18}O in samples of normal or nearly normal abundance = ± 0.003 atom %)

Exp no	Glucose monohydrate (mg)	Notatin (mg)	H_2^{18}O (ml)	Atom % excess ^{18}O in water	Duration of exp (hr)	O_2 uptake at N.T.P. (ml)	Atom % excess ^{18}O in H_2O_2
1	75	0.19	1.0	1.01	2.5	6	0.002
2	100	0.5	2.0	1.01	3.0	10	0.003
3	100	0.5	2.0	1.01	4.0	9.5	0.003

Table 2 Atom % ^{18}O in hydrogen peroxide formed on oxidation of glucose by notatin with $^{18}\text{O}_2$ in H_2^{18}O

(Experimental error of isotope analysis in samples of about 0.6 atom % excess = $\pm 0.005\%$ α Glucose monohydrate was used in Exps 1 and 2 and anhydrous β glucose in Exp 3)

Exp no	Glucose (mg)	Notatin (mg)	Water (ml)	$^{18}\text{O}_2$ evolved (calc) at N.T.P. (ml)	Temp (°)	Duration of exp (hr)	Atom % excess ^{18}O of		
							Electrolytic oxygen	Oxygen remain mg at end of incubation	Oxygen from peroxide decomposition
1	100	0.5	1	8.6	18	3.5	0.603	0.563*	0.563
2	100	0.5	1	10.2	18	3.75	0.591	0.593	0.587
3	100	0.5	1	8.6	37	1	—	0.579	0.580

* This dilution was due to an air bubble accidentally admitted during electrolysis. The presence of air was confirmed by an ion beam in the spectrometer at mass 40, showing the presence of argon.

Experiments with $^{18}\text{O}_2$ If the oxidation of glucose by notatin is carried out with isotopically enriched oxygen and ordinary water, the oxygen evolved from the hydrogen peroxide has the same isotope composition as the gaseous oxygen used (Table 2). Both sets of experiments prove conclusively that the oxygen atoms of the hydrogen peroxide produced in the notatin reaction, are not derived from the solvent, but from the oxygen of the gas phase.

The relative rates of oxidation of α and β glucose

Fig. 1 shows the results obtained with α - and β -glucose respectively. In these experiments the glucose was added last to the solution in order to minimize the effect of mutarotation of the glucose. It appears that the enzyme acts on both forms, although the β form is oxidized about 1.3 times more quickly than the α form. It cannot be assumed that α -glucose is first converted to β glucose by spontaneous mutarotation and then attacked by the enzyme, since the rate of mutarotation of glucose under the conditions used is too slow, compared with that of the enzymic oxidation. Moreover, the shape of the curve does not indicate that the rate of oxidation of α glucose increases with time. It was considered possible that the enzyme catalyses the mutarotation of glucose. This was tested in the following manner.

Anhydrous α glucose (0.54 g) was dissolved in 25 ml of water which had been boiled to remove O_2 and then quickly cooled to 20°, to 10 ml. of this solution was added 0.5 ml of 0.2% notatin and 7.5 ml. of water, whilst the control was made up to the same volume with water. Both samples

were transferred to polarimeter tubes and the changes of optical rotation observed for 4 hr. The following $[\alpha]_D$ values were obtained for the control solution: +92.5° (22.5 min.), +87.5° (31.5 min.), +68.5° (74.5 min.), +55.4° (300 min.). The sample containing notatin had the following $[\alpha]_D$ values: +76.2° (23 min.), +69.1° (32 min.), 55.5° (75 min.), +51.7° (300 min.). There thus seemed to be a definite difference between the two solutions. However, it was found

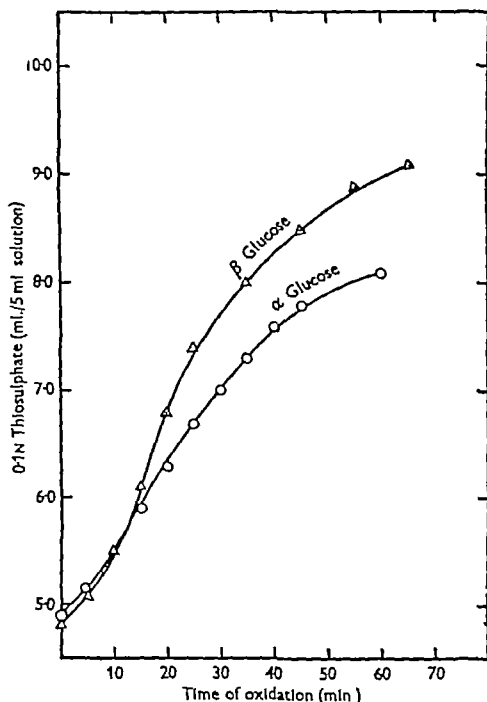


Fig 1 The relative rates of oxidation of α and β glucose by notatin. Anhydrous α or β glucose (600 mg) was added to 60 ml solution containing M acetate buffer pH 5.7 (4 ml.), catalase (0.5 ml.) and notatin (0.4 mg). Oxidation was at 22° for the times indicated by the abscissae, 0.1N I_2 (10 ml.) and 0.1N NaOH (15 ml.) were then added to 5 ml. samples. After standing for 5 min., the I_2 not used up was titrated with 0.1N $Na_2S_2O_3$ after the addition of 2.5 ml. 5% H_2SO_4 .

that the notatin had brought about the oxidation of about 3–4% of the glucose. The specific rotations of gluconic acid and the δ lactone are smaller than that of α glucose and this will account for a small part of the difference. Moreover, the formation of free gluconic acid and the consequent change of pH is bound to affect the non enzymic mutarotation of glucose. However, the slight oxidation found cannot explain the results obtained.

There is thus suggestive, but no conclusive evidence that notatin catalyses the mutarotation of glucose and that α glucose is converted enzymically to β glucose before oxidation can take place.

The nature of the primary product of oxidation

Comparison between the development of acidity and the disappearance of glucose. Gluconic acid is a

moderately strong acid. Thus gluconic acid solutions prepared by treatment of calcium D gluconate hydrate with the calculated amount of oxalic acid had the following pH values: 0.001M, 3.18; 0.01M, 2.71; 0.1M, 2.25. A 0.1M solution half neutralized with 0.1N NaOH had a pH of 3.66. The pK of gluconic acid must therefore be about 3.6–3.7. It is to be expected therefore that the formation of a small amount of free gluconic acid will, in the absence of buffering, reduce the pH to below 3.5. The pH optimum for notatin is about 5.6 (Keilm & Hartree, 1948a) and the rate of oxidation falls off rapidly from pH 4 to 3.0 to become quite small at higher acidities. Muller (1928) had already found that a decrease of pH and increase of base binding occurs in the notatin reaction and this retards the action of the enzyme. It was thought, therefore, that gluconic acid itself is the first product of this oxidation. However, it can be shown that the development of acidity is not part of the oxidation process, but occurs later and is almost certainly not catalysed by the enzyme. This is clearly shown by the following experiments.

Oxidation with a large amount of enzyme, but without buffer. β Glucose (80 mg) was added to a solution (total vol. 15 ml) containing 2.4 ml 0.2% notatin and 0.5 ml catalase. Oxidation was allowed to proceed for 8 min., by that time 84% of the glucose had been oxidized. The initial pH was 5.4 and at the end of the oxidation 3.55. The solution was now cooled to 20° and left to stand under N_2 . The pH changes were as follows: 15 min after the addition of enzyme, 3.15; 20 min, 2.99; 40 min, 2.75; 60 min, 2.55. There was no further oxidation during the second part of the experiment.

Oxidation in the presence of a small amount of buffer. In this experiment the amount of base in the acetate buffer was equivalent to 10% of the glucose used. β Glucose (0.54 g) was dissolved in a solution (total vol. 30 ml) containing 3 ml 0.12N acetic acid, 3 ml 0.1N NaOH, 0.3 ml catalase solution and 3 ml 0.2% notatin. Oxidation was allowed to proceed for 0.5 hr. During that time the pH had fallen from 5.58 to 4.29 and 94% of the glucose had been oxidized. The solution was again cooled to 20° and the pH changes noted for the following 15 hr. During the second part of the experiment a further 3% of the glucose was oxidized. The pH changes were as follows: Times (in hr) from the beginning of the experiment are given in brackets: 4.15, (0.8), 4.00, (1.1), 3.88, (1.5), 3.78, (1.75), 3.69, (2.1), 3.55, (2.7), 3.38, (4.0), 3.27, (5.0), 3.08, (8.0), 3.02, (16.0).

In this experiment the pH did not fall below 3.0 owing to the buffering effect of the gluconate ion, sodium acetate and gluconic acid must have reacted to give acetic acid and sodium gluconate.

* May, Weissberg & Herrick (1929) found a value of 3.78 for the pK of gluconic acid.

Measurement of free and total gluconic acid Table 3 shows that the primary product is a substance which does not titrate as an acid under the conditions used for the estimation of free gluconic acid, but can be estimated under the more vigorous conditions employed for 'total' gluconic acid. The most reasonable assumption is that this substance is a lactone. The relative proportion of free gluconic acid is small in the beginning of the oxidation, but increases rapidly, after most of the oxidation is finished. This is in accordance with the pH measurements reported above. The converse, of course, applies to the lactone which is measured by the difference between total and free gluconic acid.

Table 3 *Comparison of the rate of formation of free and total gluconic acid during the oxidation of glucose by notatin.*

(α Glucose (0.540 g) was dissolved in 60 ml. water containing 0.41 mg notatin and 0.5 ml catalase. Oxidation proceeded at 24°. 3 ml. samples were withdrawn at suitable intervals and titrated for free and total gluconic acid as described in the text. The very small blank values were subtracted.)

Time after addition of glucose (min)	Percentage of glucose originally present converted into		Ratio of total/free gluconic acid
	(a) Free gluconic acid	(b) Total gluconic acid	
10	0.5	10.9	21.8
20	1.0	17.9	17.9
30	1.6	24.0	15.0
60	5.1	42.4	8.3
90	9.3	52.5	5.6
128	19.0	59.3	3.2
160	35.0	75.1	2.1
1080	84.2	100.1	1.18

Polarimetric measurements

The experiments so far reported indicated that notatin converts glucose to a lactone or some other neutral substance which is slowly converted to gluconic acid. A more definite identification of this primary product was achieved by following the changes in optical rotation. δ Gluconolactone has an initial $[\alpha]_D$ of +66.2° (Nef, 1914; Isbell & Frush, 1933) which falls over 24 hr to +8.8°, whilst γ gluconolactone reaches an equilibrium value much more slowly. Fig. 2 shows that the primary product of the notatin reaction resembles δ gluconolactone very closely, the slight differences between the mutarotation curves found here and those reported by Nef (1914) or by Isbell & Hudson (1932) are caused by the fact that in experiments with pure δ lactone all the lactone is present at zero time, while in the enzymic experiments it is being formed from glucose during the first 20–30 min of the experiment. The small amount of glucose left at the end of the experiment (6.5 and 7% respectively)

explains the relatively high equilibrium figure after 24 hr, which was +12.1° instead of +8.8° found by the other workers.

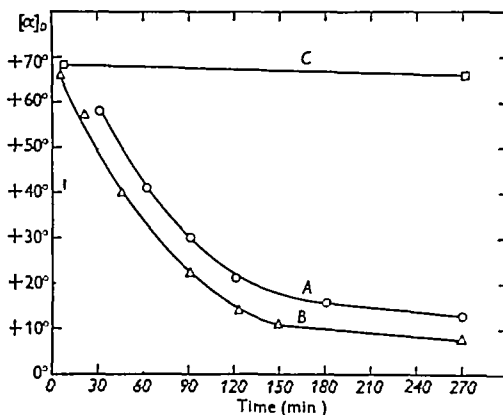


Fig. 2 *Comparison of the mutarotation of the product of the oxidation of β glucose by notatin with the mutarotation of δ and γ gluconolactone.* Curve A gives the $[\alpha]_D$ values (calculated in terms of gluconolactone) of the oxidation product. Curve B is the mutarotation curve of δ gluconolactone, and curve C is that of γ gluconolactone. The data for the two lactones were obtained from the figures given by Nef (1914), Isbell & Hudson (1932) and Isbell & Frush (1933). The oxidation was carried out as follows: Anhydrous β glucose (600 mg) was added to 30 ml of a solution containing notatin (4.8 mg) and catalase solution (0.4 ml). Oxidation proceeded for 25 min at 30°, when 93% of the glucose had been oxidized. The solution was then filtered and the rotation measured.

DISCUSSION

The isotope experiments prove conclusively that notatin catalyses the transfer of hydrogen from glucose to the oxygen of the gas phase. The fact that there was no isotope exchange between $H_2^{18}O$ and $^{16}O_2$ on shaking with notatin is confirmatory evidence that this enzyme does not produce a direct 'activation' of molecular oxygen, similar to the 'activation' of hydrogen produced by the enzyme system of *Proteus vulgaris* (Hoberman & Rittenberg, 1943).

At first, the most likely substrate of notatin appeared to be the hydrate of the aldehyde form of glucose—which could be dehydrogenated to the free gluconic acid in the manner suggested by Wieland (1932). However, Goto & Titani (1941) have shown that at room temperature, but not at 100°, the rate of exchange of glucose with $H_2^{18}O$ is very slow and that mutarotation takes place without exchange of oxygen. Since reversible hydration of glucose necessarily involves oxygen exchange on C_1 , it follows that such an uncatalysed hydration is slow at room temperature. Thus, if the actual substrate of dehydrogenation were a glucose aldehyde hydrate,

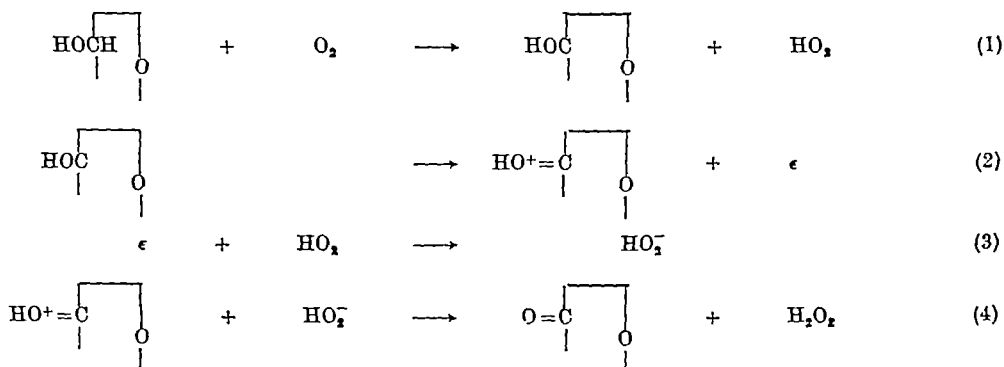
it would have to be assumed that notatin, in addition to its oxidizing action, also catalyses the conversion of ring forms of glucose to the open aldehyde form and/or the hydration of the aldehyde. Such a rather complicated hypothesis becomes unnecessary in view of the experiments reported now.

The fact that the development of acidity is not directly associated with the enzymic process and can be made to take place almost entirely after the oxidation has stopped, shows that the primary product is not gluconic acid. This is also shown by the comparison of the formation of 'free' and 'total' gluconic acid. From the polarimetric experiments it can be deduced that the neutral primary product is δ gluconolactone. It thus follows that the substrate of notatin is glucopyranose and not the free aldehyde.

about 35 times faster with β than with α glucose (Isbell & Pigman, 1933). Oxidation with hypochlorite, on the other hand, is faster with α - than with β glucose (Jeannes & Isbell, 1941).

*Detailed mechanism of the
hydrogen transfer*

In the reaction under discussion, two protons and two electrons are transferred from glucopyranose through the flavoprotein to molecular oxygen. This could occur in two successive steps, each involving the transfer of one electron, this mechanism may be pictured as follows (leaving out the intermediate reactions involving the prosthetic group of the enzyme).



hyde, or its hydrate, or one of the two glucofuranoses. If the aldehyde were the substrate, the primary product would be gluconic acid, whilst the oxidation of a furanose would have yielded the γ lactone.

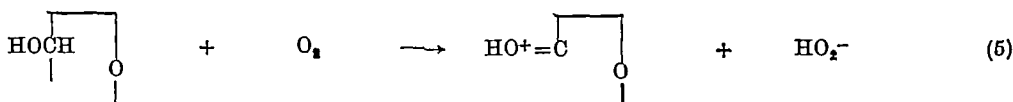
The results reported here may suggest that notatin acts on both α and β glucopyranose, though the rate is definitely faster with the β isomeride. However, suggestive evidence was obtained that notatin catalyses the mutarotation of α glucose, and it is very probable that mutarotation catalysed by notatin precedes oxidation.* Keilin & Hartree (1948a) have recently examined the substrate specificity of notatin in some detail. They have found that mannose and xylose are oxidized at about 1% of the rate found for glucose, and galactose is also attacked, albeit more slowly. It is of interest that the action of bromine on glucose in a buffered, weakly acidic medium also yields δ gluconolactone and not gluconic acid (Isbell & Hudson, 1932; Isbell, 1932). However, in this reaction the rate is

* Keilin & Hartree (private communication) have independently discovered the effect of notatin on the mutarotation of α glucose and have made this problem the subject of an extensive investigation.

In the first step the glucopyranose loses a proton and an electron to give two free radicals, $\text{HO}_2\cdot$ and a radical derived from glucose, the latter then loses a second electron to form a carbonium ion which is stabilized by resonance with an oxonium ion. A prototropic reaction between the oxonium ion and the peroxide anion then completes the oxidation. Riboflavin and flavoproteins can exist in the form of fairly stable free radicals (Haas, 1937; Michaelis & Schwarzenbach, 1938), and preliminary experiments carried out here appear to show that notatin can catalyse the reaction between titanous chloride and iodine, presumably by the mechanism suggested by Shaffer (1933, 1936). It can therefore be assumed that notatin may be an intermediate in reactions involving the transfer of one electron by being itself transformed into a free radical. However, there is no evidence for even the transient existence of a free radical derived from glucose, and the known facts are equally compatible with the assumption that the oxidation of glucose by notatin occurs by an ionic mechanism.

According to this concept the essential step in the reaction is the transfer of a hydride ion, i.e. a proton with two electrons, from the glucopyranose to mole-

cular oxygen, and thus the reactions (1), (2) and (3) are telescoped into one



This is followed by reaction (4) as above. The shift of a hydride ion has been postulated as the mechanism of other redox reactions such as a Cannizzaro reaction (viz Hammett, 1940). No facts are available which would enable us to decide which of the two interpretations is correct. In any case, the distinction may be somewhat unreal, since the ionic mechanism may be considered to represent an extreme case of the free radical mechanism in which reactions (2) and (3) are infinitely fast compared with reaction (1).

SUMMARY

1 By using water and molecular oxygen labelled with ^{18}O it is shown that the oxygen atoms of the hydrogen peroxide produced in the oxidation of glucose by notatin are derived from molecular

oxygen. It is concluded that notatin catalyses the transfer of hydrogen from glucose to oxygen.

2 A comparison of the rate of oxidation of glucose by notatin and the development of acidity shows that the first product of the enzymic action is a neutral substance. Polarimetric measurements indicate that this is δ gluconolactone which changes by a non-enzymic reaction into gluconic acid.

3 The mechanism of the transfer of two hydrogen atoms of glucose to oxygen is discussed. Two alternative interpretations are proposed, one involving a free radical mechanism and the other a simultaneous transfer of two electrons as the rate determining step.

The authors wish to thank Prof D Keilin, F.R.S., for putting at their disposal highly purified notatin, Miss M G French and Miss B Wiltshire for assistance in the chemical work and Mr G Dickinson for help with the mass spectro meter.

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An Apparatus for the Micro-electrolysis of Water

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(Received 15 July 1949)

In tracer work using D and ^{18}O it is often desirable to be able to decompose small amounts of water. This is particularly the case with ^{18}O , where rather limited amounts of ^{18}O are available as H_2^{18}O . In a study of the mechanism of the action of notatin (Bentley & Neuberger, 1949) the isotope was required as oxygen gas, an apparatus was devised in which quantities of the order of 1.5 ml. of water may be satisfactorily electrolysed and the oxygen collected in a vacuum system.

each of a roll (1 sq in.) of platinum foil spot-welded via a short length of platinum to the tungsten wires *A* (1 mm diam.) sealed into each arm of the U tube. In operation, degassed H_2^{18}O (about 1.5 ml.) containing a little Na_2SO_4 was placed in the U tube, and the apparatus evacuated with stopcocks *B* and *C* closed. (All stopcocks were greased with Apiezon vacuum grease, grade L.) By cautious manipulation of stopcock *B* the water was allowed to rise just to the barrel. Stopcock *B* was then closed, and approximately 200 V d.c. applied to the electrodes, so that the current flowing was about 0.1 amp. Hydrogen produced in the

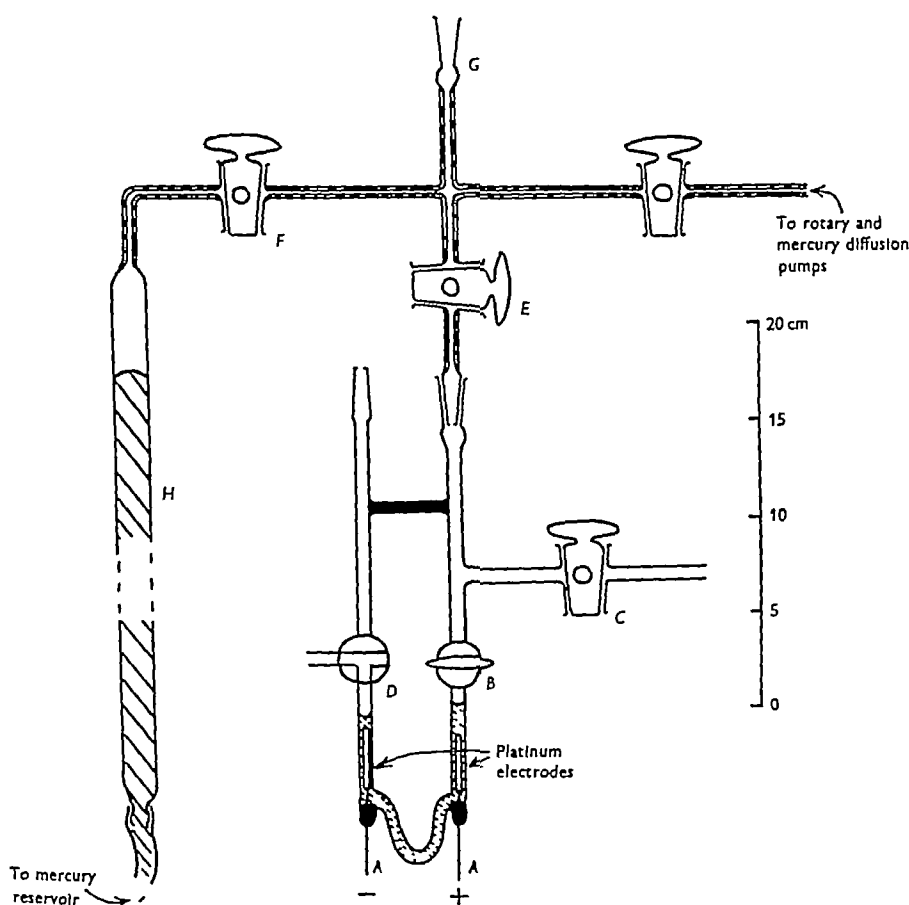


Fig 1 An apparatus for the micro electrolysis of water

EXPERIMENTAL

The apparatus (Fig 1) was constructed in pyrex glass. 6 mm. tubing was used for the cell, and 3 mm. capillary for the rest of the vacuum system. The electrodes consisted

cathode limb was allowed to escape, or could be collected separately depending on the position of the 3 way stopcock *D*. The oxygen evolved was collected under the barrel of stopcock *B*, and as the level of water in this limb diminished, stopcock *B* was cautiously opened so that the oxygen

escaped into the vacuum system and the water level was restored. It was necessary to cool the cell during electrolysis and for this purpose it was wound with heavy gauge copper wire, the ends of which dipped into an ethanol CO_2 bath. As this cooling was still insufficient, the cold ethanol was splashed over the cell with a small brush. With a current of 0.11 amp, 1.6 ml of $^{16}\text{O}_2$ at N.T.P. were evolved in 5 min.

When electrolysis was complete, the oxygen was expanded into the Toepler pump (*H*) with stopcocks *E* and *F* opened. With stopcock *E* closed it was then pumped into a previously evacuated apparatus, attached to the vacuum line through the standard joint *G* (B10). With water of 0.575 atom % excess ^{18}O in the cell (determined by equilibrium

with CO_2 and carbonic anhydrase as described by Bentley, 1948), oxygen gas of 0.579 atom % excess ^{18}O was obtained.

SUMMARY

An apparatus is described in which quantities of the order of 1.5 ml of water may be electrolysed, and the evolved oxygen (or hydrogen) collected in a vacuum system.

The author is indebted to Miss M. G. French for experimental assistance and to Mr G. Dickinson for assistance with mass spectrometric analyses.

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Growth Factors for *Corynebacterium Diphtheriae*

4. PREPARATION FROM YEAST

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(Received 15 February 1949)

In an earlier paper, Chattaway, Happold & Sandford (1944) showed that certain gravis (and intermedius) strains of *Corynebacterium diphtheriae* required an unidentified growth factor when cultivated on a medium of known composition containing pimelic, nicotinic and pantothenic acids (Mueller, 1937*a, b*; Evans, Handley & Happold, 1939*a, b*). This factor, present in liver, was shown to be dialysable and resistant to proteolytic enzymes. Its properties were consistent with the presence in its molecule of both acid and basic groups probably in the form of an α amino acid component, since the activity was destroyed by ninhydrin.

The factor appeared to have no direct connexion with any of the unidentified growth factors for *Lactobacillus casei*, cf. Dolby, Happold & Sandford (1944), but showed some similarity to streptogenin, which was prepared by Woolley (1941) from various purified proteins, and is required by certain strains of *Streptococcus haemolyticus*. The present paper reports the separation and concentration from yeast of a number of factors similar in many respects to the liver factor of Chattaway *et al.* (1944).

EXPERIMENTAL

Method of microbiological assay

Organism. The strain of *C. diphtheriae* used for assessing the potency of preparations was a gravis strain, subtype Dundee (Barton). In all cases the growth was observed

in $6 \times \frac{5}{8}$ in test tubes containing the medium outlined below.

Inocula. The strain was maintained on Löffler slopes in the refrigerator and subcultures were made on blood agar plates. A sufficient amount of an 18 hr subculture was introduced into 10 ml of sterile water to produce a faint turbidity at the surface. This suspension was dispersed by shaking and one drop (approx. 0.03 ml) used for inoculation.

Basal medium. The basal medium employed, shown in Table 1, differed from that described by Chattaway *et al.* (1944). Certain amino acids hitherto omitted were included in order to bring its composition more nearly into line with a casein hydrolysate medium on which the organism grows profusely. In the early experiments it was found that test cultures occasionally grew in the basal medium. This was traced to an impurity in the aspartic acid, which was then omitted without alteration in the response of the organism to the factor. Pimelic acid was also omitted since the organism was provided with preformed biotin.

Determination of growth. The organism used grows exclusively as a pellicle under the conditions of test, a fact which enables a visual assay of growth to be made, and which precludes all other methods with the exception of a determination of the N_2 content of a whole culture. This latter method was discarded since an adequate expression of growth could be made in terms of arbitrary degrees of pellicle formation. Ten degrees of growth were recognized, ranging from one given a numerical value of 4, corresponding to a heavy pellicle over the surface of the medium, through 3, 2, 1.5 and 1, at which value the whole surface of the medium was covered with a thin film of growth, down to degrees of growth given the numerical values 0.5, 0.2, 0.1, 0.05 and 0.02, the last being a just discernible trace. Growth was determined after 72 hr incubation at 37°. This method

Table 1 Basal medium for the growth of *Corynebacterium diphtheriae* (Dundee)
(Quantities for 1 l)

	mg		μg
Glycine	100	Biotin	1
DL-Alanine	400	p Aminobenzoic acid	50
L-Valine	700	Riboflavin	100
L-Leucine	500	Nicotinic acid	200
L-Isoleucine	100	Calcium pantothenate	100
L-Proline	400	Pyridoxine	600
L-Hydroxyproline	20		
DL-Phenylalanine	200	2M sodium lactate (from B D H	20 ml
DL-Aspartic acid	400	A R lactic acid)	
DL-Glutamic acid hydrochloride	2200	Na ₂ HPO ₄ 12H ₂ O	1 g
DL-Serine	200	K ₂ HPO ₄	0.5 g
L-Tyrosine	300	FeSO ₄ 7H ₂ O	160 μg
DL-Methionine	100	NaCl	9 g
D Arginine	400	MgSO ₄ 7H ₂ O	0.7 g
L-Histidine (base)	400		
L-Lysine hydrochloride	600	Salts solution	10 ml
L-Tryptophan	400		
L-Cystine	800	CuSO ₄ 5H ₂ O 0.05 g	} in 50 ml water
DL-Threonine	50	ZnSO ₄ 7H ₂ O 0.04 g	
		MnCl ₂ 4H ₂ O 0.02 g	

The medium was adjusted to pH 7.4 and 0.05 ml. of 0.5% CaCl₂/5 ml. of medium added aseptically after autoclaving

Table 2 The activity of yeast fractions as growth promoting substances for *Corynebacterium diphtheriae*

Fraction	$\alpha =$	Concentration of additions (10^{-2} ml/ml medium)									
		1	2	3	4	5	6	7	8	9	10
		(Activity numbers)									
Hydrolysate after removal of HCl (dry weight 250 mg /ml.)	17	100	17	1	0	0	—	—	—	—	
Filtrate from norite	67	100	33	17	7	3	0	—	—	—	
Filtrate after removal of inorganic material	33	100	100	67	50	33	7	0	—	—	
After removal of lead as phosphate	0	17	67	100	67	33	17	1	0	—	
Filtrate from phenylhydrazine containing some phenylhydrazine	0	0	100	100	67	50	33	17	7	—	
Final extract after purification from phenylhydrazine and acetic acid (dry weight 170 mg /ml)	33	67	100	100	100	100	100	67	33	17	

has proved useful since several factors are capable of causing variation in the maximum growth obtained in a test and an approximate numerical expression of the results permits the values obtained in each series of tests to be expressed as a percentage of the maximum growth obtained at that time. These values, termed activity numbers, can then be compared in tests carried out at different times.

Preparation of active concentrate

Choice of material. Previous work had been carried out on liver residues from the preparation of the antipermeous anaemia factor (the factory treatment of which was unknown to us), later samples were found to be entirely without activity presumably due to some alteration in technique. Other sources, therefore, had to be explored. Both casein hydrolysates and yeast hydrolysates contain sufficient factor for growth of the organism, but it was decided to use yeast as large quantities were available.

Fresh brewer's yeast was usually employed, but one sample of dried baker's yeast showed similar activity when worked up in the same manner. Brewer's yeast was obtained within 24 hr. of draining and was not steamed, longer periods of standing resulted in autolysis and loss of activity. The active material is an essential growth factor for *Strep faecalis* R and *Lb casei* when these organisms are

grown on amino-acid media. Material has also been prepared from human urine which is active for the above strain of *C. diphtheriae*.

Preparation of active extracts. Fresh brewer's yeast (2 kg.) was made into a slurry with 8 l. of 2N HCl, boiled rapidly for 15 hr., filtered, and the residue washed with 1 l. of water. PbO was added to the combined filtrate and washings to bring it to pH 2.8-3.0, the precipitate removed and the filtrate and washings shaken with charcoal (nonts 200 g./l.) for 2 hr., and filtered. The nonts was washed with water, the washings added to the filtrate and the pH adjusted to 6.5 with Na₂PO₄ in saturated solution. The filtrate, after the removal of lead phosphate, was concentrated to about 4 l. using capryl alcohol as an antifoam agent. To remove sugar impurities (mainly mannose) hydrated sodium acetate (2 kg.) and phenylhydrazine hydrochloride (200 g.) were added in 4 l. of water, and the phenylhydrazones filtered off 18 hr. later. Most of the excess phenylhydrazine was removed from the filtrate in the usual way with benzaldehyde (25 g.), the remainder by nine extractions with 1/3 vol. of ether. Acetic acid was removed by acidification with H₂SO₄ to pH 3, followed by repeated extraction with ether. The solution was then frozen repeatedly, and the successive crops of sodium sulphate crystals removed. The solution was concentrated in

vacuo below 40°, to about 1.2 l, treated with ethanol to a concentration of 40% (v/v), and the pH brought to 6.5 with baryta. The solution (Y 47) after removal of BaSO₄ had a dry weight of 170 mg/ml and was active at 1.7×10^{-5} µg/ml of medium. Table 2 gives the activities of fractions at various stages throughout the concentration.

Properties of yeast concentrate Y 47

Analytical figures are shown in Table 3. The preparation contained an appreciable quantity of free amino acids, and also peptides, indicated by a positive biuret reaction. No histidine, tyrosine, arginine, tryptophan or free amines were present. Application of the reaction of Elson & Morgan (1933) showed the presence of material akin to amino sugars but not identical with glucosamine.

Table 3 *Analyses of yeast extract Y 47*

(Dry weight of Y 47 = 170 mg/ml)

Material determined	Content (mg/100 mg Y 47)
Total N (Kjeldahl)	6.26
NH ₃ N	2.33
α NH ₂ N (ninhydrin)	0.92
S	Nil
Total P	0.30
Inorganic P	0.03
Carbohydrate as glucose (Hagedorn & Jensen)	53.2
Carbohydrate as glucose (Somogyi)	33.5

Inactivation (a) Hydrolysis Prolonged hydrolysis with 6N HCl considerably reduced activity. Acid hydrolysis did not affect the α amino N value appreciably.

(b) Ninhydrin Refluxing Y 47 with 1/3 saturated KH₂PO₄ (20 ml) and ninhydrin (500 mg) for 15 min, removal of excess ninhydrin with alumina (225 mg), refluxing for a further 15 min and extraction of the pigments produced with CHCl₃ resulted in complete destruction of the active material.

(c) Nitrous acid Treatment of Y 47 (0.8 ml) with NaNO₂ (300 mg) and 0.4N HCl (10 ml.) overnight at room temperature resulted in complete loss of activity.

(d) Osazone formation The filtrate after removal of phenylhydrazones from Y 15 (5 ml) by treatment in the cold with phenylhydrazine hydrochloride (0.4 g) and hydrated sodium acetate (2 g) was boiled with glacial acetic acid (1 ml) for 15 min. This treatment resulted in a partial inactivation.

Identification of the active material of Y 47 with a number of ninhydrin positive components

Many of the techniques, examined before the above scheme for separation of the active material from yeast was perfected, showed defects which proved of interest in the further study of the extract. Attempts to remove the considerable amounts of sugar present by the use of 80% ethanol, baryta, methanol, pyridine, dialysed iron, calcium chloride and basic lead acetate were all discarded owing to a partition of activity between the two phases, and extraction of Y 47 with *p*-cresol had a similar effect.

These results might be explained as the simple partition of a single factor, or by the presence of more than one factor. Attempts were therefore made to fractionate Y 47 to find if the activity was associated with one or more of the components.

The complete destruction of activity by treatment with ninhydrin suggested that the active components were amino acids or peptides. Two-dimensional partition chromatograms of Y 47 on paper, using phenol and collidine, showed the presence of twelve ninhydrin positive compounds (see Fig. 1). Of these, five have been identified, by their relative positions and by the similarity of their individual positions, with aspartic acid, glutamic acid, serine, glycine and isoleucine. The remainder which could not be correlated with known amino acids have been assigned the symbols, S1, S2, S3, P1, P2, P3 and P4. The two spots S2 and S3 were very weak, apparently indicating a low concentration, while P2 was present in high concentration.

Chromatograms obtained after hydrolysis in 6N-HCl for 18 hr at 120° in a sealed tube showed that P1, P2, P3 and P4 are peptide in nature, since they completely disappeared from the chromatogram. Under these conditions the spots, S1, S2 and S3 were stable. This implies that they are either free amino acids or acid stable peptides. Partial acid hydrolysis (6N-HCl for 18 hr at 100°) caused the breakdown of P2 and P4, but P1 and P3 were not destroyed. Indeed, the concentration of P1 and P3, as indicated by the intensity of the chromatogram spots, increased, suggesting the possibility that they are simpler peptides derived from P2 and P4. Similar degrees of stability were observed after hydrolysis with 5% w/v ammonia for 18 hr at 100° in a sealed tube. Separation of these various amino acids and peptides was undertaken before an investigation of their nature was attempted.

Separation by ionophoresis The technique of silica gel ionophoresis devised by Consden, Gordon & Martin (1947) for the separation of amino acids and short-chain peptides was applied to Y 47. The gel was made from sodium silicate, 30 ml of a 4N solution being brought to pH 7.4 (bromothymol blue) with H₃PO₄, and the whole diluted to 400 ml. This was poured into the trough and allowed to set. The cathode perfusate was 0.125N NaH₂PO₄ and the anode perfusate 0.34N Na₂PO₄ in the ratio of 1/7, 5 ml samples of Y 47 were mixed with appropriate quantities of sodium silicate solution to make the filling for the gutter. The potential applied was generally 120 V, occasionally 60 V, and the length of the gel about 50 cm.

The electrode perfusion liquids were circulated continuously (cf. Hall, 1948), and fractions collected as they passed over the anode gel/liquid interface. After runs varying in length from 5 to 22 hr the material had separated into six distinct bands. Tables 4 and 5 give the activity, composition and mobility of these bands. Most of the activity was associated with two bands, C2/1, which moved rapidly towards the anode, and C2/4 + C2/5 which contain material remaining in or near the gutter.

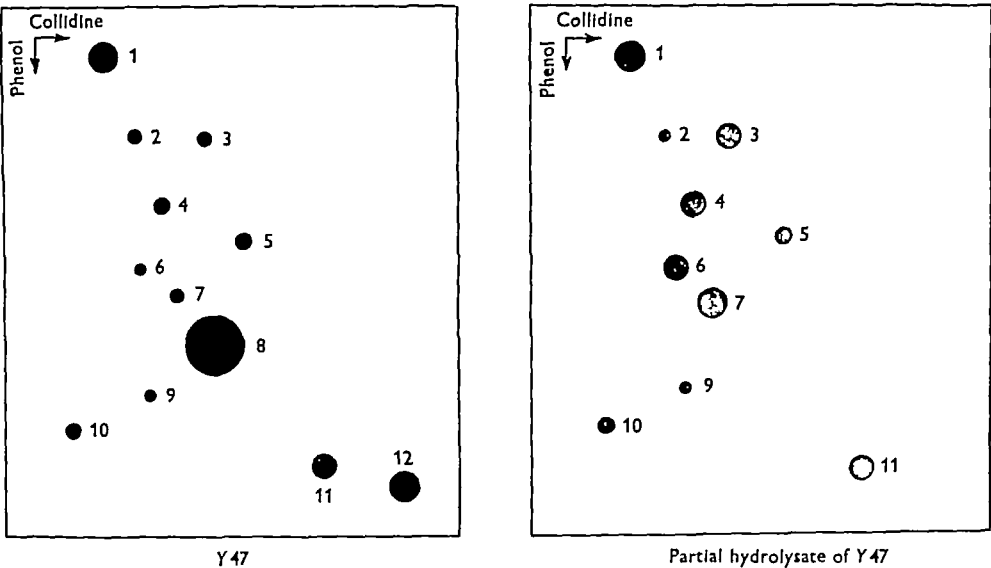


Fig 1 Phenol/collidine chromatograms of Y47 and its partial hydrolysate after heating at 100° in 6N HCl for 18 hr. The size of the circles indicates approximately the concentration of the ninhydrin positive substances, which are respectively (1) S1, (2) aspartic acid, (3) glutamic acid, (4) serine, (5) P3, (6) glycine, (7) P1, (8) P2, (9) S3, (10) S2, (11) isoleucine, (12) P4.

Table 4 Separation of ninhydrin positive components in Y47 by ionophoresis
(Separation in a silica gel, pH 7.4, potential 120 V)

Direction of movement	No of component	Distance travelled in 4 hr (mm)	Breadth of band after 4 hr (mm)	Composition determined by partition chromatography
Towards anode	C2/1	70	24	S1
	C2/2	55	5	Glutamic acid aspartic acid
	C2/3	35	6	Serine
	C2/4	15	14	P1, P2, P4, glycine
Towards cathode	C2/5	17	21	and isoleucine
	C2/6	31	6	S2

Table 5 The activity of six components of Y47 separated by ionophoresis
(C2/4 and 5 also includes any material remaining in the gutter)

Concentrations of additions, 10 ⁻² ml/ml. of medium (x)	Y47	C2/1	C2/2	C2/3	C2/4 C2/5	C2/6
		(Activity numbers)				
1	100	0	0	0	67	0
2	100	1	0	0	100	17
3	100	67	17	33	67	0
4	100	67	33	17	67	1
5	100	33	33	1	33	1
6	100	17	17	1	17	0

A similar experiment with a streptogenin concentrate, kindly supplied by Dr D W Woolley (cf Sprince & Woolley, 1945), showed no amino acids even after prolonged

application of the potential except within a range of a few millimetres of the gutter. This is in agreement with other observations that streptogenin, although containing two of the peptides present in Y47, does not contain any of the components moving more slowly in a phenol chromatogram, especially S1 (present in C2/1) and the free amino acids glycine, serine, glutamic and aspartic acids.

Separation by partition chromatography The technique devised by Dent (1947) for the separation of peptides from urine was used. Thirty-six spots of about 0.02 ml each of Y47 were applied to a paper and chromatographed in phenol for 18 hr. The total length of the run was 342 mm. The two outside traces were removed and developed with ninhydrin and the main sheet cut accordingly into ten pieces. Of these, seven were initially judged to include all the ninhydrin positive material present in Y47 while three, nos 3, 5 and 9, represented the larger interspot spaces. The breadths of the bands and their composition as indicated by running subsequently in phenol or collidine are shown in Table 6.

Table 6 *Chromatographic characteristics of the various fractions of Y 47 run in phenol*

No	Composition as judged by further chromatography	R_F of centre of band	Breadth of band (mm)
1	S1, aspartic acid, glutamic acid, serine	0.06	42
2	Glutamic acid	0.15	19
3	—	—	15
4	Serine	0.24	29
5	—	—	15
6	Glycine, P1	0.40	37
7	Glycine	0.50	46
8	P2	0.62	36
9	—	—	30
10	P4, S2, isoleucine	0.92	83

Each fraction was separately extracted with water and tested. Most of the activity was observed in fractions nos 1, 6 and 8, and slight activity in no 10.

The refractionation of these fractions by chromatography either with phenol or collidine enabled pure samples of ten of the twelve ninhydrin positive spots to be obtained, but two spots, P3 and S3, were so weak in the original chromatogram that they disappeared. Neither of these spots was associated with any detectable activity. The activity was confined to S1, P1, P2 and S2, the latter accounting for the slight activity of fraction 10, from the rest of which it could easily be separated by collidine chromatography.

Table 7 gives the activities of these fractions, none of which approaches that of the complete preparation, S1 is the most active of the components, while of the two peptides, P1 is by far the more active. All these fractions are required to produce maximum growth.

Table 7 *Activity of the unidentified fractions obtained from Y 47 by partition chromatography in phenol*

Concentration of additions, 10^{-2} ml./ml of medium (x)	Y47	S1	S2	P1	P2	P3	P4	P2
	(Activity numbers)							

1	33	100	—	67	17	3	1	—
2	67	67	17	50	7	1	0	100
3	100	33	1	17	1	0	0	100
4	100	17	0	1	0	0	0	100
5	100	1	0	0	0	0	0	33

The effect of ophio L- α -amino-acid oxidase. This enzyme system (samples of which were kindly supplied by Dr E. A. Zeller of Basle), which is apparently without action upon peptides, was used to remove unnecessary amino acids from Y47 before it was appreciated that S1 was probably an amino acid. Later experiments with the enzyme confirmed this fact. Treatment of the intact preparation with the oxidase appreciably lowered the activity and several of the spots observed in chromatograms disappeared. An acid hydrolysed sample which retained partial activity owing to the presence of S1 lost all its remaining activity when treated with the enzyme. A typical experiment is recorded in Table 8.

Table 8 *The effect of ophio-L α amino acid oxidase on the activity of Y 47 before and after hydrolysis in 6N HCl at 120° for 18 hr*

(1 ml (0.2 mg) enzyme in M/15 phosphate buffer, pH 7.4, was added to 0.1 ml Y47 or 0.1 ml hydrolysate, and incubated at 37° for 8 hr.)

Concentration of additions, 10^{-2} ml./ml of medium (x)	1 47	1 47 treated with oxidase	Y47 acid hydrolysate	Y47 hydrolysate treated with oxidase
	(Activity numbers)			
1	100	100	33	17
3	67	67	33	1
5	50	7	17	1
7	1	1	1	0

Attempted identification of some of the components

Of the active components in the preparation Y47 only P2 was present in large enough concentration to permit the use of the multispot chromatographic technique. S1, slightly contaminated with serine, aspartic and glutamic acids, could be obtained by a large scale method using a rectangular aluminium column, 18 in long and $\frac{3}{4}$ in square in cross section, with a removable side. This was packed with paper pulp, saturated with water/phenol solution and, after the addition of up to 5 ml of solution containing 1 g of solid, the chromatogram was developed with phenol run slowly through from the top. When phenol containing ninhydrin positive material appeared in the filtrate, the loose side was removed and a print of the position of the amino acid bands taken on filter paper and developed with ninhydrin. The desired portions of the column were then excised and eluted with water or extracted in a Soxhlet apparatus. Separation of relatively large quantities of amino acids into two fractions with high and low R_F values respectively was comparatively easy by this method, but the bands tended to be rather diffuse owing to non specific flow through the interstices of the paper column (see Table 9). Adequate separation of the faster running components was never practicable owing to this non specific flow, but fractions rich in any one component could be obtained.

Table 9 *The separation of activity into two gross fractions by large scale partition chromatography*

(Activity of both fractions together = 100 at all four dilutions.)

Concentration of additions, 10^{-2} ml./ml medium (x)	Aqueous extract	Phenol extract
	(Activity numbers)	
1	67	67
2	67	67
3	7	67
4	0	33

Examination of S1. S1, running very slowly both in phenol and collidine (R_F 's 0.09 and 0.15 respectively), occupies a position close to that of phosphorvserine (Dent,

1948) and phosphorylhydroxylysine (Gordon, 1948). Since serine is present in Y 47, and S2, which has slight activity, gives a spot close to that of hydroxylysine, it seemed possible that S1 might be one or other of the two phosphoryl derivatives. Estimation of the inorganic P content of S1 before and after treatment with alkaline phosphatase showed that the material contained organic P, but no change occurred in the intensities of the spot for S1, or in the activity of the preparation, nor did any more spots appear.

Examination of P2 P2, the only other component yet obtained pure in sufficient amount, was hydrolysed by 6N HCl at 120° for 18 hr and shown to consist of a polypeptide containing serine, glycine and glutamic acid, while P1 was shown to be a partial hydrolysis product of this polypeptide.

Comparison of Y 47 components with streptogenin

Examination of the streptogenin concentrate by means of a one dimensional phenol chromatogram showed that it contained only three ninhydrin positive materials (owing to the small amount available no two dimensional chromatograms were made). Two of the spots were close to P1 and P2, while the third ran slightly faster than the complex containing P4, S2, S3 and isoleucine. Table 10 shows the activity of streptogenin compared with Y 47. Direct comparison with the figures given in Table 7 for the peptides concerned is not possible, since during extraction of the peptides from the chromatograms changes occur in the salt concentration, and dry weight determinations are of no significance.

Table 10 Comparison of the activity of Y 47 and streptogenin in the growth of *Corynebacterium diphtheriae*

Additions to medium, 10 ⁻² mg./ml. (x)	Streptogenin (Activity numbers)	Y 47
2	100	100
3	50	100
4	17	100
5	0.1	100

Preliminary examination of synthetic peptides

The following dipeptides showed considerable activity at the concentrations ($\mu\text{g}/\text{ml}$) indicated: glutamylglutamic acid (0.01), glutamylglycine (0.03), glycylglutamic acid (0.1), leucylglutamic acid and glutamylalanine (from 5 to 10), alanylglutamic acid appeared to be inactive.

DISCUSSION

Several unidentified factors present in yeast extract or partial hydrolysates of protein have recently been described which appear to have some properties in common with the preparations described in this paper, the most notable being a factor required by certain strains of *Strep. lactis* (Smith, 1943), the

'sporogenes vitamin' of Peterson & Shull (1948), the factors required for maximum production of toxin by *Clostridium tetani* (Mueller & Miller, 1948) and streptogenin (Woolley, 1941). Peterson & Shull concluded from comparative assays of the two materials, using *Cl. sporogenes* and *Lb. casei* as test organisms, that their material and streptogenin were distinct. Since these two materials, and the yeast preparation described in this paper, contain a number of active components it is probable that they have one or more components in common, this is so for streptogenin and Y 47. The potency of a given preparation for a given organism may also depend upon the relative concentrations of the different components.

The preparation of a concentrate from yeast extract having considerable activity for the growth of a gravis strain of *C. diphtheriae*, type Dundee, has shown among other things the difference between this fraction and one, with similar physiological properties, obtained from liver. The active material from liver, after such mild purification as extraction with ethanol and ether, and the removal of some material on fuller's earth, was highly adsorbed on charcoal (norite) at pH 3. Crude yeast extracts, however, could be treated with norite without any significant loss in activity, and although some degree of adsorption from the final concentrate occurred, it was never complete. The concentrates, of which Y 47 is the most active so far obtained, are still mixtures of a variety of components, the activity being associated with two fractions (P1 and P2) which are probably peptide in nature and one (S1) which is stable to acid hydrolysis. The failure to extract or adsorb only the active material from Y 47 compelled us to identify the components of the mixture and then separate these in turn.

Woolley (1946) has claimed that serylglucylglutamic acid has streptogenin activity of low order and that consequently it is either a portion of a more complex compound present in streptogenin concentrates, or is similar to, but not identical with, the active material. One of the peptides (P2), common to both the streptogenin concentrate and Y 47, liberates serine, glycine and glutamic acid on hydrolysis. It is uncertain whether the amino acids are joined in the order mentioned above, but the more stable peptide P1, formed by partial hydrolysis of the tripeptide, occupies the position on a chromatogram assigned to serylglycine or glutamine by Dent (1947), the latter, however, can be excluded on the grounds of stability. It is, therefore, probable that serylglucylglutamic acid has some effect on *C. diphtheriae* metabolism, but until P2 is obtained in larger amounts, and compared with a synthetic specimen of the tripeptide, it is impossible to be certain.

A number of synthetic glutamic acid peptides have been tested for activity, some of which induced

growth in our cultures of *C. diphtheriae*. The degree of activity was in no instance as great as that observed either with Y 47 or with the more active of its constituent factors. There is, of course, the possibility that other material having marked activity is present in these synthetic peptides at concentrations too low for chromatographic identification. The peptides P 1 and P 2, however, do not make up the whole of the activity for *C. diphtheriae* S 1, the slow-moving stable material, is destroyed by the action of ophio L α amino acid oxidase and hence is presumably an amino acid. On the other hand, since according to Zeller & Maritz (1944, 1945) the presence of OH and COOH groups in amino acids prevents attack by the oxidase, the position of S 1 on chromatograms cannot be taken as indicating that it is a polycarboxylic or polyhydroxy amino acid. Since this position is not altered after treatment with phosphatase, although inorganic phosphorus is produced, one must assume that the phosphate is liberated from some component giving no ninhydrin colour. The major part of the active material of Y 47 appears to be of a peptide or amino acid nature, but there is some evidence for the existence of other active components as shown by the partial inactivation on osazone formation.

SUMMARY

1 The preparation is described of a concentrate from brewer's yeast which contains essential growth factors for certain strains of *Corynebacterium diphtheriae* gravis.

2 The activity of the whole preparation is associated with four of the twelve ninhydrin positive components.

3 Most of the activity is associated with an amino acid, S 1, and a peptide, P 1, while a second amino acid, S 2, and another peptide, P 2, have slight activity.

4 The peptide P 2 consists of serine, glycine and glutamic acid.

5 A number of synthetic glutamic acid peptides also have activity as growth factors for *C. diphtheriae*.

We wish to acknowledge the co-operation of Dr M. McMillan, in the early stages of this work, and to thank Messrs Joshua Tetley and Son Ltd for supplies of yeast, Messrs Roche Products Ltd for gifts of material and the Medical Research Council for a grant during the course of the work, and also to acknowledge the gift of a number of synthetic peptides from Dr R. Consden of the Wool Industries Research Association.

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A New Manometric Method for Determination of Respiratory Quotients

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(Received 3 January 1949)

Manometric determinations of the respiratory quotient (R.Q.) require measurements of oxygen uptake, carbon dioxide production and, in the case of glycolysing cells, changes in the bicarbonate concentration of the medium. Several methods, due to Dickens & Simer (1930, 1931), Dixon & Keilin (1933), and Summerson (1939), are available. Two identical,

or practically identical, samples of the biological material are needed when these methods are used. The initial bicarbonate concentration is obtained by acidifying and killing one sample at the beginning of the experiment, while the change in bicarbonate concentration is determined by acidifying the other sample at the end of the experiment. Intermediate

values for the rate of oxygen uptake, carbon dioxide production and for the change in bicarbonate concentration during the experiment cannot be obtained. The RQ can therefore only be measured during one experimental period. Consequently, changes in RQ with time or with variations in the environmental conditions during the experiment cannot be observed. Such changes may be determined by using an adaptation of Warburg's indirect method (Laser & Rothschild, 1939), but this method is cumbersome when multiple determinations are involved.

A new apparatus with which changes in RQ can be measured on a single sample is described in this paper.

PRINCIPLE

The principle involves the sampling of known amounts of gas and liquid from a reaction vessel. One pair of samples is simultaneously removed from this vessel and connected with subsidiary manometers, in which their contents of gaseous CO_2 and bicarbonate are independently determined. After the initial determination at zero time (t_0), this procedure is repeated three times, the reaction in the main vessel continuing undisturbed. In this way changes in total CO_2 evolved and bicarbonate concentration of the medium are determined during three experimental periods. The corresponding changes occurring in the main vessel during the time between the taking of samples can be calculated from these data and from the readings of the manometer connected to the main vessel. As each of these readings registers the algebraic sum of the O_2 uptake and total CO_2 production, the oxygen consumption during each period can be calculated and the RQ determined. In the case of cell suspensions, during each experimental period the quantity of biological material in the main vessel is diminished by the amount taken out with each previous sample of the medium. This is taken into consideration in the equations which correlate the observed pressure changes with the amount of metabolizing material involved at each step. If tissue slices or whole organisms are used, the quantity of biological material remains unaltered throughout the experiment.

DESCRIPTION OF APPARATUS

Two versions of the apparatus (A and B) have been designed, differing in detail but employing the same principle.

Apparatus A The reaction vessel is rectangular (Fig 1, A). It is connected to a differential manometer which has a compensating vessel (Fig 1, B) of about the same volume as the main vessel. The joint connecting the main vessel with its manometer is raised above the centre of the vessel, enabling the insertion of a turnable gas outlet (Fig 1, C), and of

a Keilin tube (Fig 1, D), which is hung on a short length of platinum wire fused into the inner end of the gas outlet tube. The Keilin tube can be dislodged into the main vessel at any time during the experiment by rotating the gas outlet tube. The main vessel is provided at both ends with sampling devices, on which the principle of the method is based. Both ends are identical, and only one will be described in detail. It consists of a large tap, the sampling tap (Fig 2, C), which is joined to the main vessel in such a way that its key (Fig 2, D) forms the vertical side of the vessel. The key contains four invaginations, the sampling cups, which are in communication with the main vessel. In the apparatus shown in Fig 2 the cups are in two rows vertically above each other, and at such a height that the lower ones (Fig 2, c_0 and c_2) are below the level of the liquid. They are therefore completely filled with the fluid medium, while the upper ones (Fig 2, c_0 and c_2) contain the gas in the main vessel. The key is hollow, its lower end and one side of the handle are cut open and therefore filled with water from the bath, to obtain satisfactory temperature equilibration and to eliminate buoyancy.

The sampling tap (Fig 2, C) has an upper and a lower outlet tube (Fig 2, E and F) at the levels of the sampling cups. These outlet tubes lead to joints (Fig 3, D) through which each is connected with a subsidiary differential manometer having its own compensating vessel (Fig 1, G). The complete apparatus therefore consists of five differential manometers, i.e. the main manometer and two subsidiary ones on each side. The U tube of the main manometer is in the centre, while the two subsidiary manometers on each side are so arranged that one lies inside the other. Each sampling key is provided with indicator knobs (Fig 2, G) corresponding to the positions of the sampling cups. By rotating the key outwards from the position in which all its cups communicate with the main vessel, one pair of sampling cups can be brought into communication with the corresponding outlet tubes (Fig 4). Together with the outlet tube, each sampling cup then becomes the experimental vessel of a differential manometer, in one of which the CO_2 content of the gas sample is measured, and in the other, the bicarbonate content of the fluid.

Determination of the gaseous carbon dioxide The upper outlet tube contains near its lower end a roll of filter paper (Fig 2, H) soaked with KOH which absorbs all the CO_2 in the gas sample. The change of reading in the appropriate manometer determines the amount of CO_2 absorbed. A second roll of filter paper is inserted in the same outlet tube a little higher up (Fig 2, I). This is wetted with water to adjust the water vapour tension in the subsidiary manometer to that of the main vessel. This prevents the development of pressure changes, not due to

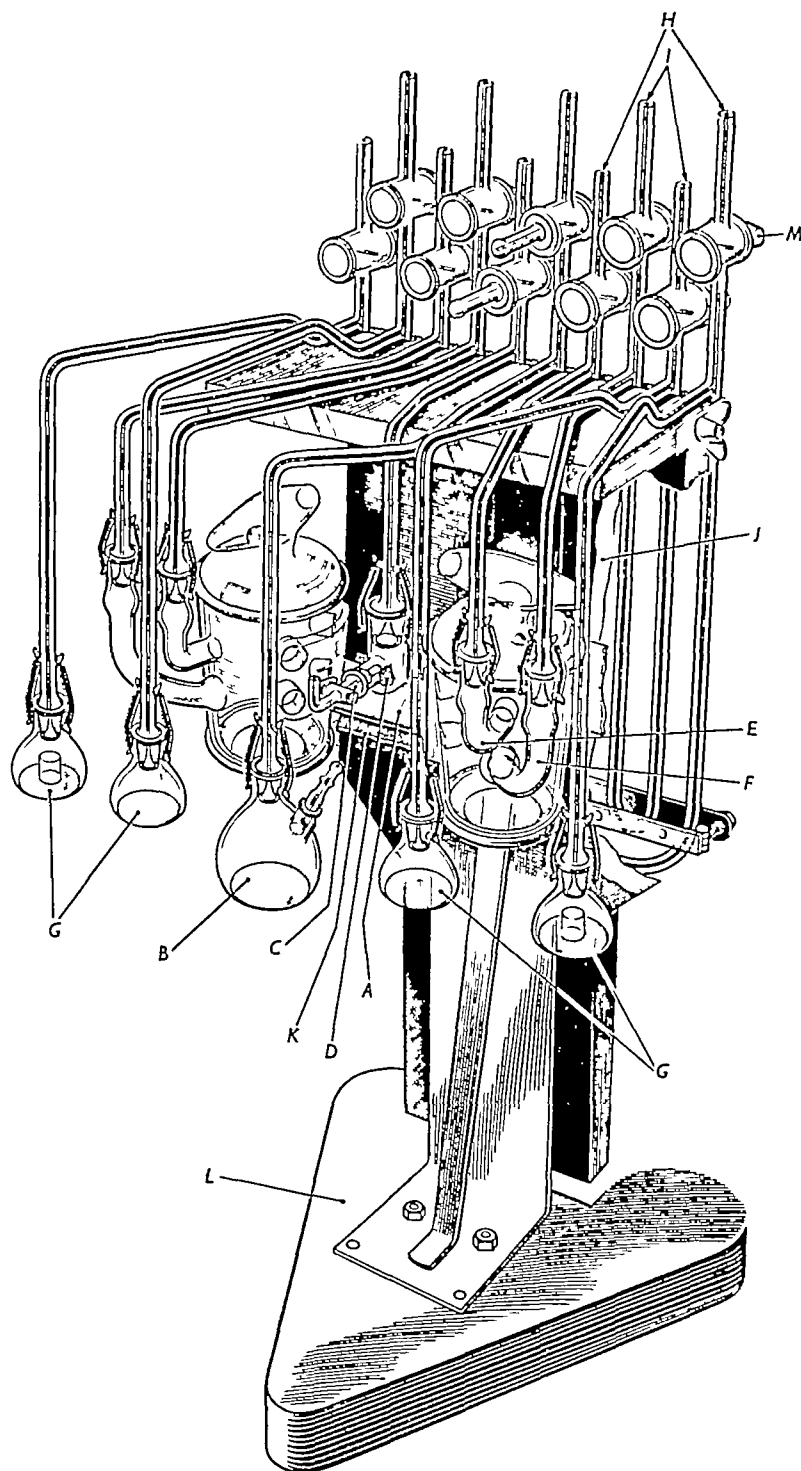


Fig 1 Rear view of assembled apparatus (Type A) *A*, main reaction vessel, *B*, main compensating vessel, *C*, turnable gas outlet, *D*, Keilin tube, *E*, upper outlet tube (for CO_2 determination of gaseous sample), *F*, lower outlet tube (for bicarbonate CO_2 determination of liquid sample), *G*, compensating vessels of subsidiary manometers, *H*, left subsidiary manometer (for determination of gaseous CO_2), *I*, left subsidiary manometer (for determination of bicarbonate CO_2), *J*, manometer board, *K*, rubber lined clamp (partly cut away) for holding main vessel, *L*, stand, *M* manometer tap Length of centre manometer, base to tap, 275 mm

CO_2 , when the gas sample from the main vessel (which is saturated with water vapour) is connected with the manometer containing KOH

Determination of bicarbonate content of the liquid sample The lower outlet tube contains known amounts of mercury (Fig 2, J) and an organic acid (Fig 2, K) When the liquid sampling cup is connected with this outlet tube, the mercury flows into the

being superimposed on the existing readings in each manometer From these readings the gaseous CO_2 and bicarbonate contents of the second pair of samples can be calculated The changes in free CO_2 and bicarbonate concentration during the interval between taking the first and second samples can be calculated if the volumes of the sampling cups are known and, in the case of the gaseous samples, the

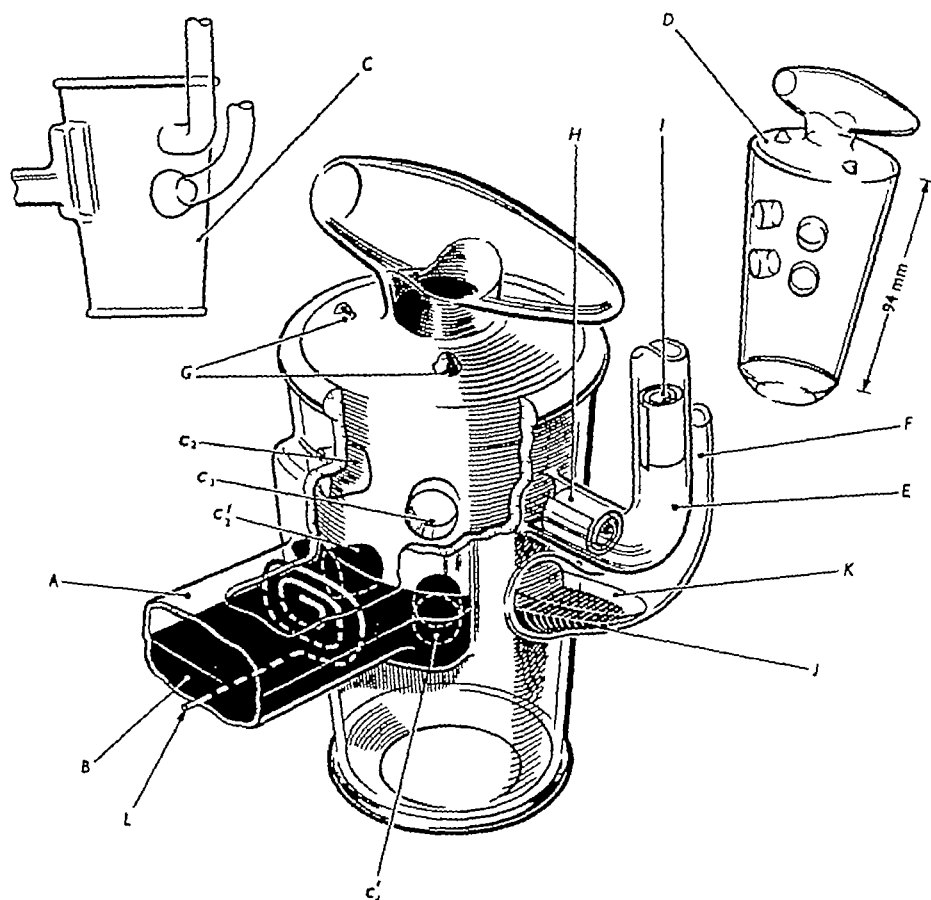


Fig 2 Section of main vessel and sampling tap (Type A) A, Main reaction vessel, B, liquid (shown in black) C, barrel of sampling tap, D, key of sampling tap, E, upper outlet tube, F, lower outlet tube, G, indicator knobs, H, filter paper for KOH, I, filter paper for water, J, mercury, K, organic acid, L, platinum coil, c_1, c_2, c_1', c_2' sampling cups

sampling cup and displaces some or all of its contents which are mixed with the acid The amount of CO_2 expelled from the bicarbonate in the sample fluid can be calculated from the change in the reading of the appropriate manometer

After an interval for CO_2 absorption and CO_2 expulsion the sampling key is turned a second time The first pair of sampling cups are thereby removed from their manometers and the next pair connected with them As neither the KOH in the upper outlet tube nor the acid in the lower one are exhausted, CO_2 absorption and expulsion are repeated, new pressures

pressure in the main manometer at the time of sampling As each sampling key contains two pairs of cups, four consecutive samples can be taken In practice the sampling keys are turned alternately, the left one at times t_0 and t_2 , and the right one at t_1 and t_3

Apparatus B This differs in one detail from apparatus A the sampling cups are situated behind each other instead of next to each other This is made possible by the introduction of two concentric keys in each sampling tap, the outer one forming a sleeve round the inner key Fig 3 shows the left

as the volume of the cup is directly proportional to the magnitude of the change which can be measured. With this in view, the gas sampling cups were made

to register small changes, are also large enough to hold the maximal pressures which can occur with 5% CO_2 in the gas space. On the other hand, the

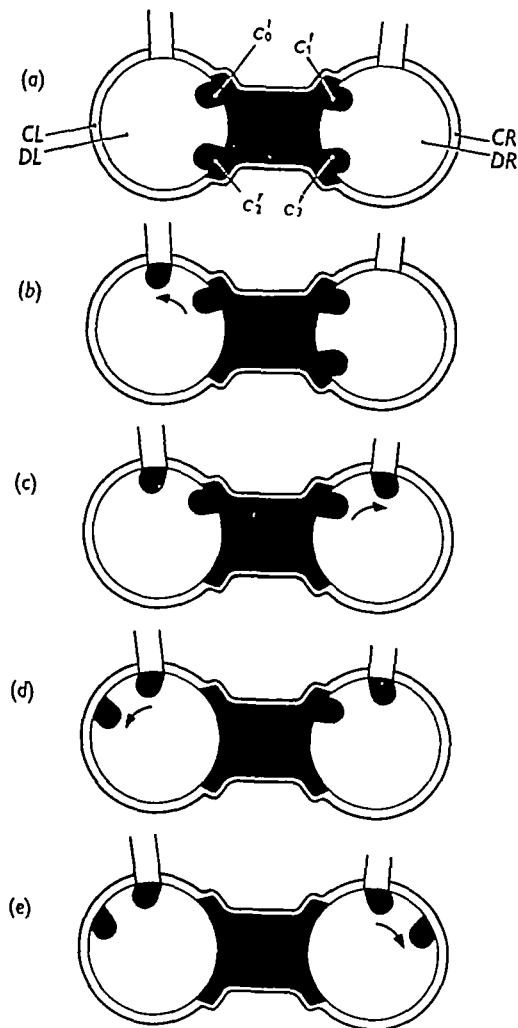


Fig 4 Schematic cross section of main vessel (Type A) at the level of the liquid (black), illustrating the sampling procedure during the course of an experiment. *CL*, barrel of left sampling tap, *DL*, key of left sampling tap, *CR*, barrel of right sampling tap, *DR*, key of right sampling tap, c'_0 and c'_2 liquid sampling cups (left), c'_1 and c'_3 liquid sampling cups (right). The arrows inside the keys indicate the direction and sequence in which the keys are turned, (a) position during temperature equilibration, (b) position from t_0-t_1 , (c) position from t_1-t_2 , (d) position from t_2-t_3 , (e) position from t_3 -constant pressure in h_3 . The same procedure applies automatically to the gaseous samples

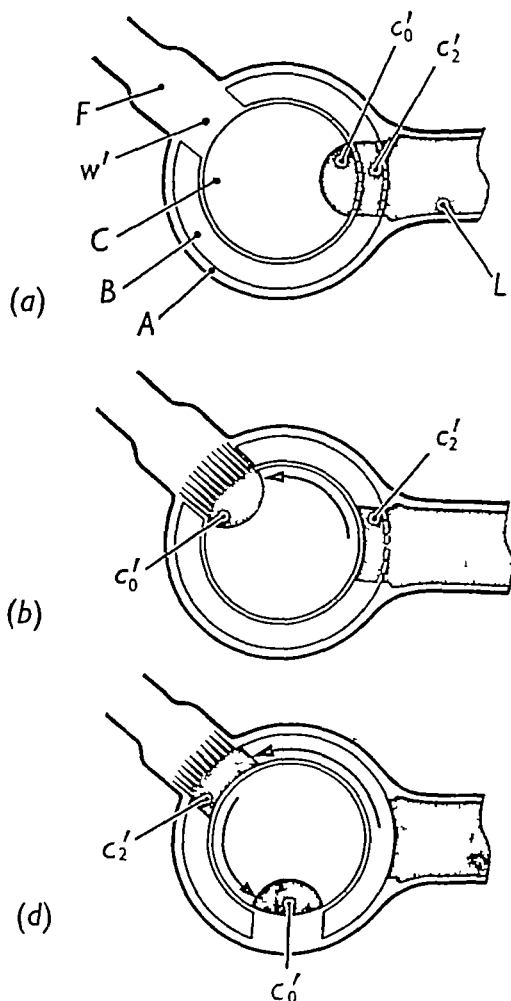


Fig 5 Schematic cross section of left side of main vessel (Type B) illustrating the sampling procedure during the course of an experiment. *A*, barrel of sampling tap, *B*, outer key, *C*, inner key, *F*, lower outlet tube, *L*, liquid in main reaction vessel, c'_0 , liquid sampling cup in inner key, c'_2 , liquid sampling cup in outer key, *w'* aperture in outer key. The arrows inside the keys indicate the direction and sequence in which the keys are turned. (a) position during temperature equilibration, (b) position from t_0-t_1 , (d) position from t_2-t_3 . Positions (c) and (e) (see Fig 4) illustrating sampling on the right side are omitted.

to hold about 1.2–1.5 ml and the liquid sampling cups 0.9–1.0 ml. Comparison of the constants shows that the subsidiary manometers for the determination of the gaseous CO_2 , while being sensitive enough

bicarbonate CO_2 manometers are not able to register more than about 200–300 μl CO_2 according to the length of the manometer capillary. This is ample when liquids of low bicarbonate concentration, such as sea water, are used. For work with Ringer-

bicarbonate (about $550 \mu\text{l CO}_2/\text{ml}$) and a liquid-sampling cup of 1 ml, the manometer would be too small. This difficulty can be overcome by decreasing the amount of liquid sampled (smaller cups in the key), or by increasing the manometer constant. There are limitations to decreasing the amount of sampled Ringer bicarbonate solution, because it is not the absolute bicarbonate content but its change which has to be determined. The constant can be increased by using a manometer fluid with high density and/or by increasing the volume of the subsidiary manometer. For this purpose an extension can be fixed on the lower outlet tube (Fig. 3, J). The manometer fluid can also be levelled after the first bicarbonate CO_2 determination. In that case the manometer only needs to register the pressure of one sample at a time. The minimum interval between sampling depends on the magnitude of metabolism and on the time taken for the expulsion of the bicarbonate CO_2 from the acidified liquid sample. Even with high bicarbonate concentrations, this was found to be complete in about 15 min. As the samples are taken from alternate sides, the effective interval can be reduced throughout the experiment to about 7.5 min/period. The interval between sampling at t_0 and t_1 can be still further reduced if the interval between t_1 and t_2 is correspondingly prolonged.

Biological application. The apparatus is applicable to the whole range of biological material which has so far been used in macromanometric work. In addition, it enables the study of many types of material for which manometric methods have been less suitable for the following reasons: (1) the absence of CO_2 in experiments with KOH interferes with their normal metabolism, (2) experiments in the presence of CO_2 using one of the existing methods are impossible, as it is not always easy to kill one sample at the beginning of the experiment, alternatively, two identical samples of the material cannot be obtained. This group includes plants, insects and their larvae, worms, small whole organs or muscle which could be stimulated *in situ*. The apparatus can also be adapted for the simultaneous determination of O_2 uptake, CO_2 production, and N_2 fixation by absorbing CO_2 in one and CO_2 and O_2 in the other outlet tube.

Metabolism without glycolysis. Many tissues and cells show no aerobic acid production. In such cases the four lower sampling cups are unnecessary. The apparatus can therefore be used without the subsidiary manometers for the determination of bicarbonate CO_2 , or a simplified apparatus can be made having only two subsidiary manometers for the determination of the gaseous CO_2 .

THEORY OF THE METHOD

Nomenclature. All symbols referring to the main manometer are expressed in capital letters and those

referring to the subsidiary manometers in lower case letters. They have the suffixes 0, 1, 2 and 3. Some of the constants have the additional suffixes O_2 or CO_2 . In the case of the main manometer the suffixes refer to the time at which the readings are taken. The amounts of gas (V_0) and of fluid (V_F) in the main vessel and the constants change at each time of sampling. If V represents the total volume of the main vessel including the eight sampling cups, and if V_{0_0} and V_{F_0} , V_{0_1} and V_{F_1} , etc. represent the gas and fluid volumes at t_0 , t_1 , etc., then H_2 is the reading on the main manometer at t_2 and $K_2 \text{ O}_2$ the O_2 constant applicable for the second time interval (t_1-t_2) based on V_{0_1} and V_{F_1} .

The gas sampling cups are called c_0 and c_2 on the left side and on the right side c_1 and c_3 , the appropriate manometer readings being h_0 and h_2 (left) and h_1 and h_3 (right). The corresponding liquid sampling cups and readings are indexed with a superscript '. Thus the liquid sampling cups are called c'_0 and c'_2 (left), c'_1 and c'_3 (right) and the readings h'_0 and h'_2 (left) and h'_1 and h'_3 (right). The volumes and constants of the subsidiary manometers also change at each time of sampling according to the volume of the sampling cup with which they become connected. As all the gaseous CO_2 which is sampled and measured in the subsidiary manometers is absorbed by KOH, the constants applicable in this case are those converting the pressure produced by a free gas and do not include the solubility factor α_{CO_2} . They are called k_0 , k_1 , k_2 and k_3 . The constants used for the determination of bicarbonate CO_2 contain the factor α_{CO_2} and are called $k'_0 \text{ CO}_2$, $k'_1 \text{ CO}_2$, $k'_2 \text{ CO}_2$ and $k'_3 \text{ CO}_2$. The suffixes in the subsidiary manometers refer to the time of sampling. Thus h'_2 and $k'_2 \text{ CO}_2$ refer to the reading and the CO_2 constant of the subsidiary manometer with respect to the liquid removed at t_2 , but read at a convenient time during the experiment when the CO_2 expulsion from bicarbonate is complete.

In determining the gaseous CO_2 only the free CO_2 in the main vessel at the time of sampling is measured. This does not include the CO_2 which has gone into solution in the fluid in the main vessel. The free CO_2 has therefore to be converted into total CO_2 by multiplication at each step with the appropriate factor $\frac{K_1 \text{ CO}_2}{K_1}$ or $\frac{K_2 \text{ CO}_2}{K_2}$ or $\frac{K_3 \text{ CO}_2}{K_3}$, where $K_1 \text{ CO}_2$, $K_2 \text{ CO}_2$ and $K_3 \text{ CO}_2$ are the CO_2 constants and K_1 , K_2 and K_3 are the corresponding constants from which the factor $V_F \alpha_{\text{CO}_2}$ has been omitted. Table 1 then applies to the readings, volumes and constants of the apparatus.

Determination of gaseous carbon dioxide. Suppose that the gas in the main vessel contains no CO_2 and that at t_0 the main manometer shows no pressure difference. Connecting the sampling cup c_0 with its subsidiary manometer will then produce no pressure

Table 1 Relations between constants of the apparatus

A Main manometer						Constants		
Time (t)	Reading	Change in reading	Gas volume	Liquid volume		K	K_{O_2}	K_{CO_2}
0	H_0	—	V_{G_0}	V_{F_0}		K_0	—	—
1	H_1	$H_1 - H_0 = \Delta H_1$	$V_{G_0} - c_0 = V_{G_1}$	$V_{F_0} - c_0' = V_{F_1}$		K_1	$K_1 O_2$	$K_1 CO_2$
2	H_2	$H_2 - H_1 = \Delta H_2$	$V_{G_0} - (c_0 + c_1) = V_{G_2}$	$V_{F_0} - (c_0' + c_1') = V_{F_2}$		K_2	$K_2 O_2$	$K_2 CO_2$
3	H_3	$H_3 - H_2 = \Delta H_3$	$V_{G_0} - (c_0 + c_1 + c_2) = V_{G_3}$	$V_{F_0} - (c_0' + c_1' + c_2') = V_{F_3}$		K_3	$K_3 O_2$	$K_3 CO_2$
B Subsidiary manometer								
Sampled time (t)	Subsidiary manometer	Outlet tube	Sampling cup	Manometer reading	Constant			
0	1	t_{ul}^*	c_0	h_0	l_0			
0	2	t_{ll}	c_0'	h_0'	$l_0 CO_2$			
1	3	t_{ur}^\dagger	c_1	h_1	l_1			
1	4	t_{lr}	c_1'	h_1'	$l_1 CO_2$			
2	1	t_{ul}	c_2	h_2	l_2			
2	2	t_{ll}	c_2'	h_2'	$l_2 CO_2$			
3	3	t_{ur}	c_3	h_3	l_3			
3	4	t_{lr}	c_3'	h_3'	$l_3 CO_2$			

t = outlet tube (Figs 2 and 3, E and F), ul = upper left, ur = upper right, ll = lower left, lr = lower right

* In apparatus B this becomes $t_{ul} + w_l$

† In apparatus B this becomes $t_{ur} + w_r$

change in that manometer. On the other hand, if at t_0 a pressure exists in the main manometer, the introduction of the gas volume contained in c_0 into the subsidiary manometer will produce a pressure difference depending on the pressure at which the gas is introduced and on the constant of the subsidiary manometer. The change of reading in the subsidiary manometer will be

$$\Delta_0 = H_0 \frac{K_0 c_0}{V_{G_0} l_0}$$

Similarly, on sampling gas at t_1, t_2 and t_3 , the following changes of pressure in the subsidiary manometers will be produced, depending on the pressure in the main vessel at these times

$$\Delta_j = H_j \frac{K_j c_j}{V_{G_j} l_j} \quad (j = 1, 2 \text{ or } 3).$$

Theoretically the Δ equations need two corrections to account for the solubility of gases other than CO_2 in the liquid contained in the subsidiary manometer, and the changes in this factor with variations in the relative amounts of these gases due to the O_2 uptake during the experiment. Both correction factors are small as the amount of liquid in the subsidiary manometer is small (0.4 ml) and the gases concerned, mainly O_2, N_2, CO and H_2 , have a low solubility. l_0 differs from $l_0 O_2, l_0 N_2$, etc. only by 0.001. Apart from the magnitude of this factor, it can safely be omitted as the CO_2 changes are calculated as differences and are unaffected by the omission of the same correction factor in all four determinations. However, because of the variations in the ratios of these gases during the experiment, this factor alters very slightly during the experiment. The Δ values calculated from the above equations differ by less than 0.1 mm from the theoretical figure for an O_2 uptake of 500 μ l in the main vessel. Both factors can therefore be omitted without affecting the accuracy of the result.

If the sampled gas contains CO_2 , the Δ values have to be subtracted from the corresponding readings to evaluate the amount of CO_2 present. If $x_0 CO_2$ is the amount of CO_2 present in c_0 ,

$$x_0 CO_2 = (h_0 - \Delta_0) l_0,$$

or

$$x_0 CO_2 = h_0 l_0 - H_0 \frac{K_0 c_0}{V_{G_0}}$$

In this equation the term l_0 , contained in Δ_0 , cancels out. Similarly, the terms l_1, l_2 and l_3 contained in Δ_1, Δ_2 and Δ_3 , cancel out in the subsequent equations for $x_1 CO_2, x_2 CO_2$ and $x_3 CO_2$ which will contain the factors

$$H_j = \frac{K_j c_j}{l_j} \quad (j = 1, 2 \text{ or } 3)$$

Thus

$$x_1 CO_2 = h_1 l_1 - H_1 \frac{K_1 c_1}{V_{G_1}}$$

The absorption of CO_2 by KOH produces a negative reading in the subsidiary manometer. To conform with the usual sign conventions the equations for $x_0 CO_2$ and $x_1 CO_2$ are converted into

$$x_0 CO_2 = H_0 \frac{K_0 c_0}{V_{G_0}} - h_0 l_0, \quad (1)$$

$$x_1 CO_2 = H_1 \frac{K_1 c_1}{V_{G_1}} - h_1 l_1 \quad (2)$$

$x_1 CO_2$ represents the total amount of CO_2 in c_1 which includes the amount of CO_2 present in c_1 at t_0 . Therefore the change of CO_2 in c_1 between t_0 and t_1 is

$$\Delta x_1 CO_2 = x_1 CO_2 - x_0 CO_2 \frac{c_1}{c_0} \quad (3)$$

On connecting c_2 with its subsidiary manometer a new pressure is superimposed on the existing reading h_0 . This does not remain as an unaltered fraction of the new reading h_2 , because part of the system in which the pressure h_0 has previously been produced, i.e. c_0 , has been removed. The fraction of h_0 included in h_2 is*

$$\frac{h_0 h_0}{h_2} \frac{t_{ul}}{t_{ul} + c_0}$$

Similarly, on determining $x_{3:CO_2}$, h_3 will contain a fraction of the previous reading h_1 , this fraction being†

$$\frac{h_1 h_1}{h_3} \frac{t_{ur}}{t_{ur} + c_1}$$

These factors have to be subtracted from h_2 and h_3 for the evaluation of $x_{2:CO_2}$ and $x_{3:CO_2}$

$$x_{2:CO_2} = H_2 \frac{K_2 c_2}{V_{G_2}} - \left[h_2 \frac{h_0 h_0}{h_2} \frac{t_{ul}}{t_{ul} + c_0} \right] h_2$$

$$\text{or } x_{2:CO_2} = H_2 \frac{K_2 c_2}{V_{G_2}} + h_0 \frac{h_0 t_{ul}}{t_{ul} + c_0} - h_2 h_2, \quad (4)$$

$$\Delta x_{2:CO_2} = x_{2:CO_2} - x_{1:CO_2} \frac{c_2}{c_1}, \quad (5)$$

$$x_{3:CO_2} = H_3 \frac{K_3 c_3}{V_{G_3}} + h_1 \frac{h_1 t_{ur}}{t_{ur} + c_1} - h_3 h_3, \quad (6)$$

$$\Delta x_{3:CO_2} = x_{3:CO_2} - x_{2:CO_2} \frac{c_3}{c_2} \quad (7)$$

The Δ -values for CO_2 so far obtained, (3), (5), (7), are those occurring in c_1 from t_0 to t_1 , in c_2 from t_1 to t_2 , and in c_3 from t_2 to t_3 . These have to be converted into the corresponding changes/ml of suspension, if cell suspensions are used (the amount of which changes with each time of sampling) or changes/mg of weight or dry weight, if material such as tissue slices or whole organisms are used (the amount of which remains constant throughout the experiment). The latter equations are suffixed *a*. Thus

ΔX_{CO_2} /ml cell suspension

$$\Delta X_{j:CO_2} = \Delta x_{j:CO_2} \frac{V_{G_j}}{V_{F_j}} \frac{K_j c_{CO_2}}{K_j} \quad (j = 1, 2 \text{ or } 3), \quad (8), (9), (10)$$

ΔX_{CO_2} /unit weight

$$\Delta X_{j:CO_2} = \frac{\Delta x_{j:CO_2}}{wt} \frac{V_{G_j}}{c_j} \frac{K_j c_{CO_2}}{K_j} \quad (j = 1, 2 \text{ or } 3) \quad (8a), (9a), (10a)$$

* In apparatus B this expression becomes

$$\frac{h_0 h_0}{h_2} \frac{t_{ul}}{t_{ul} + w_l + c_0}$$

† In apparatus B this expression becomes

$$\frac{h_1 h_1}{h_3} \frac{t_{ur}}{t_{ur} + w_r + c_1}$$

Determination of bicarbonate carbon dioxide Equations 11–14 express the bicarbonate concentrations as μl CO_2 /ml fluid. They are calculated for the case in which the amount of mercury introduced into the subsidiary manometers on each side (Fig. 2, J) is greater than the volume of fluid with which the mercury changes place on sampling at t_0 and at t_1 . In this case the total volume of fluid in c'_0 and c'_1 is forced up into the lower outlet tubes of the sampling taps and remains there during the determination for the samples from c'_2 and c'_3 . This facilitates the calculation of $x_{2:B}$ and $x_{3:B}$

$$x_{0:B} = \frac{h_0 h_0 c_{CO_2}}{c_0}, \quad (11)$$

$$x_{1:B} = \frac{h_1 h_1 c_{CO_2}}{c_1}, \quad (12)$$

$$x_{2:B} = \frac{h_2 h_2 c_{CO_2} - h_0 h_0 c_{CO_2}}{c_2}, \quad (13)$$

$$x_{3:B} = \frac{h_3 h_3 c_{CO_2} - h_1 h_1 c_{CO_2}}{c'_3} \quad (14)$$

The change in bicarbonate concentration corresponds to acid production or glycolysis (X_G)

ΔX_G /ml cell suspension

$$\Delta X_{1:G} = x_{0:B} - x_{1:B}, \quad (15)$$

$$\Delta X_{2:G} = x_{1:B} - x_{2:B}, \quad (16)$$

$$\Delta X_{3:G} = x_{2:B} - x_{3:B}, \quad (17)$$

ΔX_G /unit weight

$$\Delta X_{1:G} = \frac{V_{F_1}}{wt} (x_{0:B} - x_{1:B}), \quad (15a)$$

$$\Delta X_{2:G} = \frac{V_{F_2}}{wt} (x_{1:B} - x_{2:B}), \quad (16a)$$

$$\Delta X_{3:G} = \frac{V_{F_3}}{wt} (x_{2:B} - x_{3:B}) \quad (17a)$$

Respiratory carbon dioxide The respiratory CO_2 (resp CO_2) is obtained by deducting the glycolytic CO_2 from the total CO_2 for each period, thus $\Delta X_{j:CO_2}^{resp}$ /ml cell suspension or per unit weight

$$\Delta X_{j:CO_2}^{resp} = \Delta X_{j:CO_2} - X_{j:G} \quad (j = 1, 2 \text{ or } 3) \quad (18), (19), (20), (18a), (19a), (20a)$$

Determination of oxygen uptake The O_2 uptake is calculated from the readings of the main manometer. The following equations apply

ΔX_{O_2} /ml cell suspension

$$\frac{\Delta H_1}{V_{F_1}} = \frac{\Delta X_{1:O_2}}{K_{1:O_2}} + \frac{\Delta X_{1:CO_2}}{K_{1:CO_2}},$$

whence

$$\Delta X_{j:O_2} = \left[\frac{\Delta H_j}{V_{F_j}} - \frac{\Delta X_{j:CO_2}}{K_{j:CO_2}} \right] K_{j:O_2} \quad (j = 1, 2 \text{ or } 3) \quad (21), (22), (23)$$

ΔX_{O_2} /unit weight

$$\frac{\Delta H_1}{wt} = \frac{\Delta X_1 O_2}{K_1 O_2} + \frac{\Delta X_1 CO_2}{K_1 CO_2},$$

whence

$$\Delta X_{j O_2} = \left[\frac{\Delta H_j}{wt} - \frac{\Delta X_j CO_2}{K_j CO_2} \right] K_j O_2 \quad (j = 1, 2 \text{ or } 3)$$

(21a), (22a), (23a)

The respiratory quotient is given by the ratios in Table 2

Table 2 Calculation of respiratory quotients

(The numbers refer to equation numbers)

t	R Q with	
	Cell suspensions	Tissue slices, or whole organism
0-1	18/21	18a/21a
1-2	19/22	19a/22a
2-3	20/23	20a/23a

Table 3 Symbols to be introduced into equations 1-23

Symbol	Equation	Intro duced for	Symbol	Equation	Intro duced for
A	1	$\frac{K_0 c_0}{V_{G_0}}$	O	11	$\frac{K'_0 CO_2}{c'_0}$
B	2	$\frac{K_1 c_1}{V_{G_1}}$	P	12	$\frac{K'_1 CO_2}{c'_1}$
C	4	$\frac{K_2 c_2}{V_{G_2}}$	Q	13	$\frac{K'_2 CO_2}{c'_2}$
D	6	$\frac{K_3 c_3}{V_{G_3}}$	R	13	$\frac{K'_3 CO_2}{c'_3}$
E_t	4	$\frac{k_0 t_{ui}}{t_{ui} + c_0}$	S	14	$\frac{K'_3 CO_2}{c'_3}$
E_r	6	$\frac{k_1 t_{ur}}{t_{ur} + c_1}$	T	14	$\frac{K'_1 CO_2}{c'_1}$
F	3	$\frac{c_1}{c_0}$	U	21	$\frac{K_1 O_2}{V_{F_1}}$
G	5	$\frac{c_2}{c_1}$	V	21	$\frac{K_1 O_2}{K_1 CO_2}$
H	7	$\frac{c_2}{c_1}$	W	22	$\frac{K_2 O_2}{V_{F_2}}$
I	8	$\frac{V_{G_1} K_1 CO_2}{V_{F_1} c_1 K_1}$	X	22	$\frac{K_2 O_2}{K_2 CO_2}$
J	9	$\frac{V_{G_2} K_2 CO_2}{V_{F_2} c_2 K_2}$	Y	23	$\frac{K_3 O_2}{V_{F_3}}$
K	10	$\frac{V_{G_3} K_3 CO_2}{V_{F_3} c_3 K_3}$	Z	23a	$\frac{K_3 O_2}{K_3 CO_2}$
L	8a	$\frac{V_{G_1} K_1 CO_2}{c_1 K_1}$			
M	9a	$\frac{V_{G_2} K_2 CO_2}{c_2 K_2}$			
N	10a	$\frac{V_{G_3} K_3 CO_2}{c_3 K_3}$			

Simplification of calculations The equations contain a number of factors which are constant for a given set of experimental conditions at a given temperature. These factors can be calculated and expressed by symbols (Table 2), the introduction of which facilitates subsequent calculation. The equations can then be written down in the following way, those suffixed *a* referring to unit weight

$$x_0 CO_2 = AH_0 - h_0 k_0, \quad (I)$$

$$x_1 CO_2 = BH_1 - h_1 k_1, \quad (II)$$

$$\Delta x_1 CO_2 = (II) - F (I), \quad (III)$$

$$x_2 CO_2 = CH_2 + E_1 h_0 - h_2 k_2, \quad (IV)$$

$$\Delta x_2 CO_2 = (IV) - G (II), \quad (V)$$

$$x_3 CO_2 = DH_3 + E_r h_1 - h_3 k_3, \quad (VI)$$

$$\Delta x_3 CO_2 = (VI) - H (IV), \quad (VII)$$

$$\Delta X_1 CO_2 = I (III), \quad (VIII)$$

$$\Delta X_2 CO_2 = J (V), \quad (IX)$$

$$\Delta X_3 CO_2 = K (VII), \quad (X)$$

$$\Delta X_1 CO_2 = \frac{L (III)}{wt}, \quad (VIIIa)$$

$$\Delta X_2 CO_2 = \frac{M (V)}{wt}, \quad (IXa)$$

$$\Delta X_3 CO_2 = \frac{N (VII)}{wt}, \quad (Xa)$$

$$x_{0B} = Oh_0, \quad (XI)$$

$$x_{1B} = Ph_1, \quad (XII)$$

$$x_{2B} = Qh_2, -Rh_0, \quad (XIII)$$

$$x_{1B} = Sh_3 - Th_1, \quad (XIV)$$

$$\Delta X_{1\sigma} = (XI) - (XII), \quad (XV)$$

$$\Delta X_{2\sigma} = (XII) - (XIII), \quad (XVI)$$

$$\Delta X_{3\sigma} = (XIII) - (XIV), \quad (XVII)$$

$$\Delta X_{1\sigma} = \frac{V_{F_1}}{wt} (XI - XII), \quad (XVa)$$

$$\Delta X_{2\sigma} = \frac{V_{F_2}}{wt} (XII - XIII), \quad (XVIa)$$

$$\Delta X_{3\sigma} = \frac{V_{F_3}}{wt} (XIII - XIV), \quad (XVIIa)$$

$$\Delta X_1^{resp} CO_2 = (VIII) - (XV), \quad (XVIII)$$

$$\Delta X_2^{resp} CO_2 = (IX) - (XVI), \quad (XIX)$$

$$\Delta X_3^{resp} CO_2 = (X) - (XVII), \quad (XX)$$

$$\Delta X_1^{resp} CO_2 = (VIIIa) - (XVa), \quad (XVIIIa)$$

$$\Delta X_2^{resp} CO_2 = (IXa) - (XVIa), \quad (XIXa)$$

$$\Delta X_3^{resp} CO_2 = (Xa) - (XVIIa), \quad (XXa)$$

$$\Delta X_1 o_1 = U\Delta H_1 - V \text{ (VIII)}, \quad (\text{XXI})$$

$$\Delta X_2 o_2 = W\Delta H_2 - X \text{ (IX)}, \quad (\text{XXII})$$

$$\Delta X_3 o_3 = Y\Delta H_3 - Z \text{ (X)}, \quad (\text{XXIII})$$

$$\Delta X_1 o_2 = \frac{\Delta H_1 K_1 o_2}{wt} - V \text{ (VIIIa)}, \quad (\text{XXIa})$$

$$\Delta X_2 o_2 = \frac{\Delta H_2 K_2 o_2}{wt} - X \text{ (IXa)}, \quad (\text{XXIIa})$$

$$\Delta X_3 o_2 = \frac{H_3 K_3 o_2}{wt} - Z \text{ (Xa)} \quad (\text{XXIIIa})$$

Equations I-VII and XI-XIV apply equally to cell suspensions and tissue slices or organisms. Equations VIII-XXIII and VIIIa-XXIIIa express the values for total CO_2 output, glycolytic CO_2 , respiratory CO_2 and O_2 uptake as $\mu\text{l}/\text{ml}$ cell suspension or $\mu\text{l}/\text{unit}$ weight for each separate period of sampling. In the case of non glycolysing tissues, the formulae for bicarbonate CO_2 (XI-XIV), glycolysis (XV-XVII) and for the determination of the respiratory CO_2 (XVIII-XX) do not apply. Equations VIII-X give directly the respiratory CO_2 , and only equations I-X and XXI-XXIII need be calculated. Once the symbols have been calculated, the evaluation of results is obtained from equations I-XXIII. If this procedure is followed, calculation of an entire experiment may be completed in about 30 min.

MANIPULATION OF THE APPARATUS RESULTS

The assembly and manipulation of the apparatus are simple. The wide manometer board (Fig 1, J) has at its back two slides which are arranged to fit into two neighbouring brackets on the shaker bar of the tank. The manometers are not fixed to the board, but are kept in a wooden stand which is provided with slots to fit the U shaped parts of the manometers. The main vessel is kept in a wooden block. When assembling the apparatus the vessel is not put on to the manometers as is usual, but the manometers, with the compensating vessels in position, are slid into their joints after the vessel has been fixed in a rubber lined clamp (Fig 1, K) at the back of the board. An alternative type of clamp secures the vessel round the sampling taps to allow illumination of the vessel. All compensating vessels contain water, those connected to the subsidiary manometers for the determination of gaseous CO_2 having in addition KOH on filter papers in their centre wells, to obtain the same water vapour tension as in the experimental side of the manometer. The sampling keys are greased and so inserted into the taps that all sampling cups are in open communication with the main vessel. The lower rolls of filter paper are then inserted into the upper outlet tubes and soaked with 0.2 ml 10% KOH. The upper rolls of filter

paper are next inserted and wetted with 0.2 ml water. (A standard size of Whatman no 2 filter paper is being used, $15 \times 75 \text{ mm}$, the volume of which is determined and taken into account when calculating the constants.) Redistilled mercury (3 ml)—care must be taken that no air is trapped—and tartaric acid (0.5 ml of 0.5 N) are run into each lower outlet tube. The main vessel is then filled with the amount of suspension or fluid necessary to fill and completely cover the lower sampling cups. Tissue slices or small organisms must be prevented from lodging in the lower sampling cups. This is done by placing a platinum coil in the vessel (schematically drawn in Fig 2, L) or by putting the material in a small platinum-wire basket. Ringer bicarbonate solution should be equilibrated with 5% (v/v) CO_2/O_2 before putting it into the vessel. The gas-outlet tap is introduced, and, if necessary, the Keilin tube attached. The apparatus is assembled on a solid stand (Fig 1, L) which has two brackets fitting the slides of the manometer board. The vessel is secured in the clamp at the back of the manometer board and the central manometer inserted. The greased joint is worked in by gentle movement of the manometer and fixed with springs. If gassing is required or continuation of gassing with a CO_2 gas mixture, this is done in the ordinary way at this stage. Next, the outer and then the inner subsidiary manometers are slid into position in their joints and fixed. All manometers are secured in front of the manometer board by two rubber-lined clamps across the width of the board. The gassing is stopped, the gas outlet tap closed and the manometer transferred into the bath. If necessary, gassing can be done or continued while the manometer is shaken in the bath. In this case the gas outlet (Fig 1, C) is prolonged above the surface of the water by a length of rubber tubing. It is advisable to gas both sides of the main manometer in order to prevent excessive movement of the manometer fluid, which, with the narrow capillary (area 0.25 sq mm), may affect later readings because of the slow draining of traces of manometer fluid. Temperature equilibration is proceeded with as in any differential manometer. The subsidiary manometers do not need gassing. It is, however, important to shut all manometers simultaneously with the compensating vessel of the main vessel. This is done when the position of the fluid in the main manometer is adjusted before the reading at t_0 . In this way small temperature differences during the experimental period do not affect the later readings of the samples.

The four subsidiary manometers should show no pressure differences up to the moment when they are connected with one of the sampling cups. The position of the sampling cups during the course of the experiment is diagrammatically shown in Fig 4. At t_0 subsidiary manometers 1 and 2 (left) and the

main manometer (H_0) are read. The left sampling tap is immediately turned in order to connect the first pair of sampling cups (c_0 and c'_0) with their manometers. At t_1 subsidiary manometers 3 and 4 (right) and the main manometer (H_1) are read. The right sampling tap is then turned to connect c_1 and c'_1 with their manometers. At t_2 subsidiary manometers 1 and 2 (their pressures, h_0 and h'_0 , having in the meantime become stationary) and the main manometer (H_2) are read. The left tap is then turned once more to connect the next pair of sampling cups (c_2 and c'_2) with their manometers. At t_3 subsidiary manometers 3 and 4 (h_1 and h'_1) and the main manometer (H_3) are read and the right tap turned once more to connect the last pair of sampling cups (c_3 and c'_3) with their manometers. All readings of the main manometer (H_0 , H_1 , H_2 and H_3) have by now been taken. The remaining readings, h_2 and h'_2 (left), h_3 and h'_3 (right), are taken when the pressures in these manometers are constant.

In apparatus *B* the left inner key is turned at t_0 , the right inner key at t_1 , the left outer key at t_2 and the right key at t_3 (Fig. 5). The readings are taken in the way described for apparatus *A*.

CALIBRATION

The most accurate way of calibrating the apparatus is with mercury. In the main vessel the total volume has to be determined (including the eight sampling cups), and the separate volume of each sampling cup. The constants of the subsidiary manometers can also be obtained experimentally. It is not advisable to do so in the first instance if no previous experience of the handling of the apparatus has been gained. However, it may be useful to check the calibrated constants in the following ways.

Constants of the subsidiary manometers for the determination of gaseous carbon dioxide. The main vessel contains water and both rolls of filter paper in the subsidiary manometers on each side are wetted with water (no KOH to be included). A positive pressure is produced in the main manometer by a gas other than CO_2 . This corresponds to the reading H_0 . In this case $H_0 = H_1 = H_2 = H_3$. The left and right sampling keys are turned once

$$l_j = \frac{H_j K_j c_j}{V_{O_2} h_j} \quad (j = 1 \text{ or } 2)$$

The manometer fluid in the subsidiary manometers is then levelled and the procedure continued

$$h_j = \frac{H_j K_j c_j}{V_{O_2} h_j} \quad (j = 2 \text{ or } 3)$$

Constants of the bicarbonate carbon dioxide manometers. A bicarbonate solution of known concentra-

tion is made up (approximately $50 \mu\text{l CO}_2/\text{ml}$) and its concentration checked by acidifying in an ordinary manometer. The bicarbonate solution is pipetted into the main vessel and the lower outlet tubes filled with mercury and tartaric acid. After temperature equilibration, the sampling taps are turned as described. The subsidiary manometers are not levelled after the first readings left and right. If the known bicarbonate concentration/ml is A ,

$$l_{j \text{ CO}_2} = \frac{A c_j}{h_j} \quad (j = 1 \text{ or } 2),$$

$$h_{2 \text{ CO}_2} = \frac{A c_2 + (h_0 l_{0 \text{ CO}_2})}{h_2},$$

$$h_{3 \text{ CO}_2} = \frac{A c_3 + (h_1 l_{1 \text{ CO}_2})}{h_3}$$

Protocols

Exp. 1 This concerned the influence of light, in air, on the metabolism of *Gastrophilus intestinalis* larvae. It was done with Mr L. Levenbook as part of a study of the influence of CO on the metabolism of *Gastrophilus intestinalis* larvae. The significance of the light effect on the ratio CO_2/O_2 is not discussed here. Apparatus type *B*, temp., 37° , constants given in Table 4. The larvae rested on wetted filter paper.

Table 4 Constants of apparatus used in Exp. 1

$K_1 \text{ O}_2 = 4.4404$	$l_0 = 0.757$		
$K_2 \text{ O}_2 = 4.30$	$l_1 = 0.8303$		
$K_3 \text{ O}_2 = 4.1472$	$l_2 = 0.6441$		
	$l_3 = 0.7360$		
$A = 0.0985$	$F = 0.9387$	$L = 49.6421$	$V = 0.9831$
$B = 0.0915$	$G = 1.10$	$M = 43.8393$	$X = 0.9785$
$C = 0.10$	$H = 0.985$	$N = 44.7451$	$Z = 0.9776$
$D = 0.0948$			
$E_l = 0.4727$			
$E_r = 0.5501$			

The main vessel contained in addition to the larvae a small metal boat, filled with water. The total volume of larvae, boat and water was adjusted to 5 ml. Wet weight of four larvae, 1.9 g.

Table 5 Manometer readings, Exp. 1

t (min.)	Experimental conditions	Main manometer		Subsidiary manometer, gaseous CO_2
		$H_0 = -0.2$	$\Delta H_1 = +21.4$	$h_0 = -14.1$
0-30	Light	$H_1 = +21.2$	$\Delta H_2 = -16.0$	$h_1 = -17.5$
30-60	Dark	$H_2 = +5.2$	$\Delta H_3 = +29.8$	$h_2 = -45.6$
60-90	Light	$H_3 = +35.0$		$h_3 = -51.2$

The readings (Table 5) give the following relationships

$$\begin{aligned}
 \text{I} \quad x_0 \text{ CO}_2 &= 0.0985 \times -0.2 + 14.1 \times 0.757 = 10.66 \\
 \text{II} \quad x_1 \text{ CO}_2 &= 0.09147 \times 21.2 + 17.5 \times 0.8303 = 16.51 \\
 \text{III} \quad \Delta x_1 \text{ CO}_2 &= 16.51 - 0.9387 \times 10.66 = 6.51 \\
 \text{IV} \quad x_2 \text{ CO}_2 &= 0.10 \times 5.2 + 0.4727 \times -14.1 + 45.6 \times 0.6641 = 23.24 \\
 \text{V} \quad \Delta x_2 \text{ CO}_2 &= 23.24 - 1.10 \times 16.51 = 5.04 \\
 \text{VI} \quad x_3 \text{ CO}_2 &= 0.09481 \times 35.0 + 0.5501 \times -17.5 + 51.2 \times 0.8303 = 31.39 \\
 \text{VII} \quad \Delta x_3 \text{ CO}_2 &= 31.39 - 0.985 \times 23.24 = 8.54 \\
 \text{VIIIa} \quad \Delta X_1 \text{ CO}_2 &= \frac{49.6421}{1.9} \times 6.51 = 170 \\
 \text{IXa} \quad \Delta X_2 \text{ CO}_2 &= \frac{43.8393}{1.9} \times 5.04 = 116.5 \\
 \text{Xa} \quad \Delta X_3 \text{ CO}_2 &= \frac{44.7451}{1.9} \times 8.54 = 200.3 \\
 \text{XXIa} \quad \Delta X_{1-\text{O}_2} &= \frac{21.4}{1.9} \times 4.4404 - 0.9831 \times 170 = -117 \\
 \text{XXIIa} \quad \Delta X_{2-\text{O}_2} &= \frac{-16}{1.9} \times 4.30 - 0.9785 \times 116.5 = -150.2 \\
 \text{XXIIIa} \quad \Delta X_{3-\text{O}_2} &= \frac{29.8}{1.9} \times 4.1472 - 0.9776 \times 200.3 = -131
 \end{aligned}$$

The results are given in Table 6

Table 6 *Results of Exp 1*

(q'_{O_2} and q'_{CO_2} represent O_2 uptake and CO_2 production/l g larvae/hr)

t (min)	Experimental conditions	q'_{O_2}	q'_{CO_2}	$\frac{q'_{\text{CO}_2}}{q'_{\text{O}_2}}$
0-30	Light	234	340	1.45
30-60	Dark	300.4	233	0.775
60-90	Light	262	400.6	1.53

Exp 2 This concerned the metabolism of 4 sheep retinæ in Ringer-bicarbonate with 0.2% glucose and 5% CO_2 /95% O_2 at 38° . Apparatus type B, constants given in Table 7. Manometer fluids paraffin (d , 0.788) in main manometer and in subsidiary manometers for determination of gaseous CO_2 , α -bromonaphthalene (d , 1.4875) in subsidiary manometers for determination of bicarbonate CO_2 . Extensions were used on the lower outlet tubes (see Fig. 3, J).

Owing to large pressure changes occurring in the subsidiary manometers for the determination of the

gaseous CO_2 at t_2 and t_3 , they were opened and levelled after the determinations of h_0 and h_1 . The expressions $E_1 h_0$ and $E_2 h_1$ in equations IV and VI, therefore, become zero and are omitted. Dry weight of retinæ, 101 mg.

Table 7 *Constants of the apparatus used in Exp 2*

$V_{F_1} = 24.0745$	$K_1 \text{ O}_2 = 3.08$	$l_0 = 0.757$	$k'_0 \text{ CO}_2 = 3.92$
$V_{F_2} = 23.2613$	$K_2 \text{ O}_2 = 2.996$	$l_1 = 0.8303$	$k'_0 \text{ CO}_2 = 3.97$
$V_{F_3} = 22.4792$	$K_3 \text{ O}_2 = 2.899$	$l_2 = 0.6641$	$k'_0 \text{ CO}_2 = 3.99$
		$l_3 = 0.7360$	$k'_3 \text{ CO}_2 = 4.07$
A = 0.1009	F = 0.9387	L = 43.4279	
B = 0.09488	G = 1.10	M = 38.8585	
C = 0.1026	H = 0.985	N = 37.5445	
D = 0.1029			
O = 4.2562	V = 0.7486		
P = 4.3398	X = 0.7496		
Q = 4.6820	Z = 0.7499		
R = 4.5999			
S = 4.6504			
T = 4.5361			

Table 8 *Manometer readings, Exp 2*

t (min)	Main manometer	Subsidiary manometers	
		Gaseous CO_2	Bicarbonate CO_2
	$H_0 = -127.4$	$h_0 = -116$	$h'_0 = +125$
0-20	$H_1 = -37.2$ $\Delta H_1 = +90.2$	$h_1 = -127$	$h'_1 = +116.6$
20-40	$H_2 = +7.8$ $\Delta H_2 = +45.0$	$h_2 = -212.5$	$h'_2 = +227.3$
40-60	$H_3 = -34.5$ $\Delta H_3 = +26.7$	$h_3 = -210.3$	$h'_3 = +216.6$

The readings (Table 8) give the following relationships

$$\begin{aligned}
 \text{I} \quad x_0 \text{ CO}_2 &= 0.1009 \times -128 + 116 \times 0.757 = 75 \\
 \text{II} \quad x_1 \text{ CO}_2 &= 0.09488 \times -37.2 + 127 \times 8303 = 101.9 \\
 \text{III} \quad \Delta x_1 \text{ CO}_2 &= 101.9 - 0.9387 \times 75 = 31.5 \\
 \text{IV} \quad x_2 \text{ CO}_2 &= 0.1026 \times 7.8 + 212.5 \times 0.6641 = 142 \\
 \text{V} \quad \Delta x_2 \text{ CO}_2 &= 142.3 - 1.1 \times 101.9 = 30.0 \\
 \text{VI} \quad x_3 \text{ CO}_2 &= 0.1029 \times 34.5 + 210.3 \times 0.7360 = 158.6 \\
 \text{VII} \quad \Delta x_3 \text{ CO}_2 &= 158.6 - 0.985 \times 142 = 18.6 \\
 \text{VIIIa} \quad \Delta X_1 \text{ CO}_2 &= \frac{43.4279}{101} \times 31.5 = 13.8 \\
 \text{IXa} \quad \Delta X_2 \text{ CO}_2 &= \frac{38.8585}{101} \times 30.0 = 11.6 \\
 \text{Xa} \quad \Delta X_3 \text{ CO}_2 &= \frac{37.5445}{101} \times 18.6 = 6.95 \\
 \text{XI} \quad X_{0B} &= 125 \times 4.2562 = 532 \\
 \text{XII} \quad X_{1B} &= 116.0 \times 4.3388 = 506 \\
 \text{XIII} \quad X_{2B} &= 227.3 \times 4.6820 - 125 \times 4.5999 = 489.3 \\
 \text{XIV} \quad X_{3B} &= 216.6 \times 4.6504 - 116.6 \times 4.5301 = 478.3 \\
 \text{XVa} \quad \Delta X_{1O} &= \frac{24.0745}{101} \times (532 - 506) = 6.23 \\
 \text{XVIa} \quad \Delta X_{2O} &= \frac{23.26125}{101} \times (506 - 489.3) = 3.84 \\
 \text{XVIIa} \quad \Delta X_{3O} &= \frac{22.4791}{101} \times (489.3 - 478.3) = 2.48 \\
 \text{XVIIIa} \quad \Delta X_{1\text{CO}_2}^{\text{resp.}} &= 13.8 - 6.23 = 7.57 \\
 \text{XIXa} \quad \Delta X_{2\text{CO}_2}^{\text{resp.}} &= 11.6 - 3.84 = 7.76 \\
 \text{XXa} \quad \Delta X_{3\text{CO}_2}^{\text{resp.}} &= 6.95 - 2.48 = 4.47 \\
 \text{XXIa} \quad \Delta X_{1O_2} &= \frac{90.2}{101} \times 3.08 - 0.7486 \times 13.8 = 7.57 \\
 \text{XXIIa} \quad \Delta X_{2O_2} &= \frac{45.0}{101} \times 2.9962 - 0.7496 \times 12.0 = 7.49 \\
 \text{XXIIIa} \quad \Delta X_{3O_2} &= \frac{26.7}{101} \times 2.899 - 0.7499 \times 6.45 = 4.44
 \end{aligned}$$

Table 9 Results of Exp 2

(Q_{O_2} , $Q_{CO_2}^{\text{resp.}}$, Q_a , metabolic quotients of O_2 , respiratory CO_2 , and glucose are in $\mu\text{l}/\text{mg}$ dry wt/hr)

t (min.)	Q_{O_2}	$Q_{CO_2}^{\text{resp.}}$	Q_a	R.Q.
0-20	22.7	22.7	18.7	1.0
20-40	22.5	23.3	11.5	1.04
40-60	13.3	13.4	7.5	1.0

Results are given in Table 9. The figures for O_2 uptake and total CO_2 production agree closely with those obtained when using the indirect method of Warburg (1924, 1925).

SUMMARY

1 The existing manometric methods of measuring the respiratory quotient allow only one determina-

tion to be made during one experimental period. So far it has not been possible to measure changes of R.Q. with time, or after alterations of the experimental conditions during the experiment. Such changes occur on addition of substrates, inhibitors, catalysts, drugs, or on fertilization.

2 A new manometric apparatus, which allows such changes to be measured on a single sample of biological material, has been made.

3 The method involves taking samples of the liquid and gaseous phases from the main vessel. Samples are taken at the beginning of the experiment and at three convenient times during the experiment, which is unaffected by the sampling procedure. The CO_2 and bicarbonate contents of the samples are

determined in subsidiary manometers. These, together with the readings of the main manometer at the times of sampling, enable the O_2 uptake, and the production of respiratory CO_2 and acid to be independently calculated for each interval between sampling. In this way changes with time in the rates of O_2 uptake, total CO_2 production and of R.Q. can be obtained.

4. Two forms of the apparatus have been designed and are described. The theory of the method is developed and a simplified way of computing the results is given.

The instruments were made by Messrs W. G. Flaig and Sons, Waterloo Road, London, N.W. 2.

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Carotenoids, Vitamin A and 7-Dehydrosteroid in the Frog (*Rana temporaria*)

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(Received 2 March 1949)

Understanding of the biochemistry of carotenoids is rudimentary except as to the part played in the animal economy by a few of them as precursors of vitamin A. In a wider sense the animal biochemistry is complicated by species differences. Thus some animals absorb and retain so little of either the hydrocarbons or their hydroxylated derivatives as to suggest that they are not needed, whilst other species absorb relatively large quantities of different carotenoids selectively and retain them distributed over various organs. For the carotenes it could be argued that they are only absorbed as such when the capacity of enzyme systems in the gut to transform them is exceeded, and that the pigments, of no special value to the body, are slowly disposed of. Such an argument, however, neglects the regular occurrence of carotenoids in eyes, yellow bone marrow and corpora lutea and is of little value in respect to the assimilation and metabolism of hydroxylated carotenoids like lutein and zeaxanthin.

As a subject for detailed study the frog has several special advantages, carotenoids (including both β -carotene and lutein) are widely distributed in the body, the species can be studied through the life cycle, as embryos, as tadpoles and as immature and mature frogs, there is a marked seasonal cycle in both sexes and the reproductive phase entails a heavy drain on body reserves, especially in the female. Moreover, as an amphibian, the frog provides a link between work on mammals and fishes. There are, therefore, numerous factors potentially capable of correlation with data on the distribution of caro-

tenoids so as to lead to hypotheses concerning the functions of these substances.

For 10–11 weeks after using up the residual yolk the tadpole is mainly vegetarian, but when metamorphosis is complete, frogs become mainly carnivorous. Adult frogs build up large fat reserves in early summer, and from June to August the testes or ovaries enlarge considerably. The testes then shrink and during the next few months vary little in size until the spermatozoa become fully ripe, the ovaries go on developing, but after spawning in March the shrunken oviducts are orange in contrast to their earlier paleness. The corpora adiposa of Amphibia are associated with the gonads, in the frog they vary from strands of orange tissue to large cream-coloured lobes. After surgical removal of one fat body the ovary on the operated side remains small compared with the other. According to March (1937) sexually mature frogs have a body length (snout-cloaca) of about 4.8 cm. and weigh about 10 g. Applied with caution, body length is a guide to age.

FAT SOLUBLE SUBSTANCES IN THE FROG

Earlier work on carotenoids and vitamin A. Lönnberg (1929) reported the presence of a 'carotene like pigment' in the skin (*Rana temporaria* and *R. esculenta*). Dietel (1933) noted the presence of lipochrome in skin, ovaries and liver, but thought it consisted wholly of carotene. Manunta (1934) found 'xanthophyll' and much carotene in the skin of *R. esculenta*. Rand (1935) observed vitamin A to be present in frog and toad livers with lipochrome pigments detectable in skin, liver, ovaries and eggs, oviducts, testes, kidneys,

lungs, spleen and fat bodies. He did not discriminate between free 'xanthophyll' and esterified 'xanthophylls' so that his analyses are incomplete. He used too few frogs for his work to be more than exploratory. Brunner & Stein (1935), using phase partition and chromatography, concluded that, in *R. esculenta*, β carotene and lutein (free or esterified) were the only carotenoid pigments present. In the fat bodies, liver and skin, the 'xanthophyll' was all ester form, but free lutein also occurred in the ovaries. Zechmeister & Tuzson (1936), starting from 491 frog livers, prepared crystalline specimens of liver carotenoids. The skin, ovaries, fat bodies and livers of fourteen females (*R. esculenta*) were quantitatively analysed by chromatographic and spectroscopic methods. Three fractions were separated: (a) epiphasic pigment, (b) free hypophasic pigment, and (c) esterified hypophasic pigment, the latter always predominating, (a) consisted of α and β -carotene, the β isomer predominating, (b) was a mixture of lutein and zeaxanthin. Many unidentified zones were observed during the chromatography, but it is not clear to what extent the substances responsible were metabolic products or *in vitro* artefacts. Other workers include Bartz & Schmitt (1936), van Eekelen (1934), Wald (1935), Ackermann (1938), Gillam (1938) and Lederer & Rathmann (1938).

Origin of ingested carotenoids Relatively little is known about the carotenoids of the various species of insects, snails and worms which form the diet of frogs, although β carotene and lutein probably preponderate. Judging from the contents of the alimentary tracts of frogs (in which chlorophyll is always present) a large proportion of the ingested carotenoid is in the form of plant material unabsorbed from the digestive tracts of insects eaten. From the point of view of carotenoid intake the frog is indirectly vegetarian to the extent that it makes use of plant pigments which have not previously entered the body proper of animals.

GENERAL PLAN OF THE PRESENT WORK

Neither the design of the experiment as a whole nor the interpretation of data is easy. If it had been possible to work on 600 frogs individually rather than in batches of twenty, the plan could have been different and the data obtained could have been treated statistically. Many of the analyses, however, would have been impossible on organs from single frogs. The choice lay between making a general survey with its attendant limitations and greatly restricting the scope of the work. On balance, taking into account the paucity of information in the field, it seemed better to secure the analytical data which time permitted on samples convenient to deal with, than to select particular variables for study on larger numbers of frogs.

EXPERIMENTAL

Animals

The frogs were obtained from a commercial supplier (Norwich district) and delivered at monthly intervals. Each

batch consisted of about twenty males and twenty females. The sexes were segregated on arrival. The frogs were weighed, killed by pithing, and dissected. The various organs, livers, kidneys, fat bodies, etc. from twenty animals of the same sex were combined and weighed. Each sample was then analysed for total fat and the fat in turn analysed quantitatively for as many of the following as seemed appropriate in each case: (a) vitamin A (free and esterified), (b) total carotenoids, (c) carotene, (d) 'xanthophyll', (e) free 'xanthophyll', mono ester and di ester. Within a batch, the work on one sex was completed before beginning on the other.

Batches of frogs were obtained from late March to December in 1946 and 1947 and some in 1948. The only part of the annual cycle which was not studied was the period of actual hibernation. Ova were obtained at all stages of maturity and tadpoles at all stages between the fertilized egg and the young frog. At various periods one of us (D.G.R.) collected animals from Hillside, near Southport, Lancashire, for additional tests. These included the tadpoles and young frogs (*R. temporaria*) and tadpoles of the natter jack toad (*Bufo calamita*).

Chemical methods

Outline Specimens of tissue were ground with sand and anhydrous Na_2SO_4 and extracted with ether. The solvent was removed and the lipid made up to volume with light petroleum. Measured volumes were taken for the determination of vitamin A, carotenoids and fat. Chromatographic methods were used to separate the components and the quantitative determinations were based on data obtained with the Beckman photoelectric spectrophotometer. Exposure of extracts to light was avoided so far as possible. The extracts were not subjected to heat treatment at any stage, except in removing solvent in the fat determination.

Material for analysis The frogs, which had been kept in tanks, supplied with water but no food, were roughly dried with blotting paper before weighing and dissecting. After weighing, the frogs were killed and dissected as quickly as possible. Before the viscera were weighed, adhering coelomic and other fluids and clotted blood were removed, the weighings were made without delay.

Solvents Diethyl ether was always freshly distilled over a little reduced Fe before use. Light petroleum (b.p. 40–60°) must be practically free from 'polar' impurities, the commercial product is variable and must either be rigorously purified or selected for chromatography. A consignment of good quality petroleum was reserved for this study. The acetone was of ordinary reagent quality and the CHCl_3 of commercial B.P. quality. Ethanol and cyclohexane were specially purified for spectroscopy.

Total fat The portion of the ethereal extract which was soluble in light petroleum was weighed after dehydration with ethanol and recorded as 'total fat'.

Extraction The tissue was quickly ground, a little at a time, in a mortar with about five times its weight of anhydrous Na_2SO_4 to which fine acid washed silver sand was added. Tadpoles were treated similarly without dissection. The well ground material was transferred to a beaker and covered with ether. After standing 1–2 hr. in the dark, the yellow extract was decanted through a sintered glass filter (G4) under suction. The solid residue was then transferred to the filter cup and washed with successive portions of ether until the filtrate became colourless. The solid was then returned to the beaker and left to stand under ether for

0.5–1 hr and filtered. A third extraction, during which the ether was boiled for a short time, yielded no more colour but removed residual vitamin A. In some cases the finely ground tissues were allowed to stand overnight under ether in the dark in a corked flask. The solution was decanted on the filter and the residue washed with ether until no more pigment came through. Skin, muscle, lungs, oviducts, oesophagus, stomach and intestines were extracted overnight, other tissues as quickly as possible. In all cases, the ether was removed by distillation under reduced pressure at room temperature. The residue was dissolved in light petroleum and made up to volume. A small amount of anhydrous Na_2SO_4 was added if the solution was not perfectly clear. A measured portion of the extract was used for direct estimation of vitamin A, the light petroleum being removed and replaced by a suitable volume of cyclohexane.

Chromatographic separation

Commercial bone meal was 'defatted' by means of boiling acetone. The fraction which then passed through a 120 British Standards Specification sieve was used for chromatography. When such material is stirred with acetone, the smallest particles do not settle readily, and if the liquid is decanted the 'fines' can be removed. This is an advantage as otherwise acetone eluates may be cloudy. Alumina (Savory and Moore) which had been in stock in the laboratory for some years and had become rather weakly adsorbent proved well suited to the separation of carotenoids. Type 'O' alumina (Peter Spence and Co.) could be reproducibly weakened by stirring in definite proportions of water.

A chromatographic tube of 18 mm internal diameter was used throughout this work. A column of bone meal 3 cm in height sufficed for the separation of vitamin A ester from the free vitamin and a 4 cm column for carotenoid separation. The adsorbent was poured dry into the tube, light petroleum was added and the bone meal stirred with a long thin metal rod to liberate trapped air bubbles. Strong suction was applied, but from then onwards the column was not allowed to run 'dry'. The initial adsorption of solutes was effected slowly under gravity alone. Development and elution were more rapid at 120–150 drops/min under suction. Small volumes of eluate were aimed at and colourless intermediate fractions obtained in carotenoid analyses were discarded. Vitamin A esters were eluted completely by 100 ml. of light petroleum and the free vitamin by means of a little acetone. Solvents were removed under reduced pressure and the residues made up to suitable volume in cyclohexane.

Carotene (in some cases contaminated with esterified 'xanthophyll') was carried through the bone meal column by not more than 25 ml. of light petroleum. A measured portion of this eluate was put through a similar adsorption tube containing a 4 cm column of alumina, here 30 ml. of light petroleum carried down all the carotene and the esterified 'xanthophyll' was washed through with acetone (10–20 ml.). The free 'xanthophyll' retained on the bone meal column was eluted with acetone. Throughout this paper 'xanthophyll' refers to a fraction consisting mainly of lutein, but possibly containing some zeaxanthin and a little cryptoxanthin.

For a more complete separation of fractions containing little vitamin A a fresh portion of extract was adsorbed on alumina, the carotenes eluted by means of light petroleum,

the 'xanthophyll' di esters by 1% of acetone in light petroleum (v/v) and the mono esters by 8% of acetone in light petroleum (v/v). In some cases the 'xanthophyll' fraction from bone meal was eluted with acetone, the solvent completely removed and replaced by light petroleum and the solution chromatographed on alumina. When necessary, di esters and mono esters can be selectively eluted from bone meal by means of 2% (v/v) and 8% (v/v) CHCl_3 in light petroleum, respectively. The final fractions were made up to volumes suitable for spectroscopic examination. In replacing one solvent by another heat treatment was avoided. The visible spectra of the three 'xanthophyll' fractions are reproduced in Fig. 1. If the extracts were saponified before chromatography, the 'xanthophyll' fraction was substantially homogeneous.

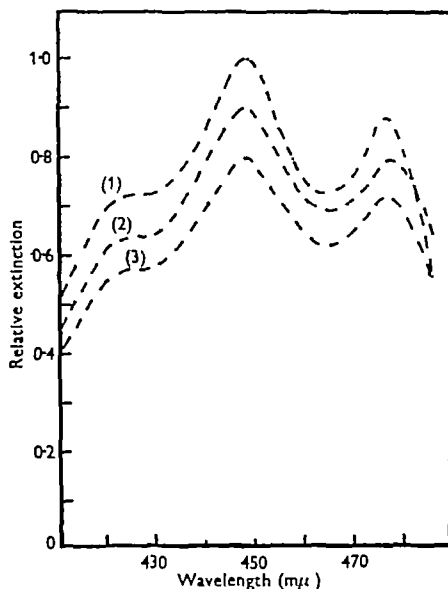


Fig. 1. Visible absorption spectra in acetone of the three forms of 'xanthophyll': (1) free xanthophyll, (2) xanthophyll di ester, (3) xanthophyll mono-ester.

Spectroscopic determinations

Vitamin A. Vitamin A was determined by the intensity of absorption at 328 $\text{m}\mu$ and the gross $E_{1\%}^{1\text{cm}}$ values were corrected for irrelevant absorption by the method of Morton & Stubbs (1946). The separation of esterified vitamin A from the free alcohol was carried out by the method of Glover, Goodwin & Morton (1947) using bone meal as chromatographic adsorbent. As a rule the amount of free vitamin A was small and was best determined by the difference between the total (corrected) and ester (corrected).

Carotenoids. The spectra of nearly all the fractions agreed very closely with those of the corresponding purified carotenoids from other sources when the criteria adopted were (a) wavelengths of maximum absorption in specified solvents, and (b) relative intensities of absorption at maxima and minima (Fig. 2). However, certain carotenoid fractions, from intestines and eyes, for example, exhibited irrelevant absorption. This was particularly noticeable in the lipids from frog eyes (Fig. 3). The colour is due mainly to 'xanthophyll' and the curve may be corrected for irrelevant

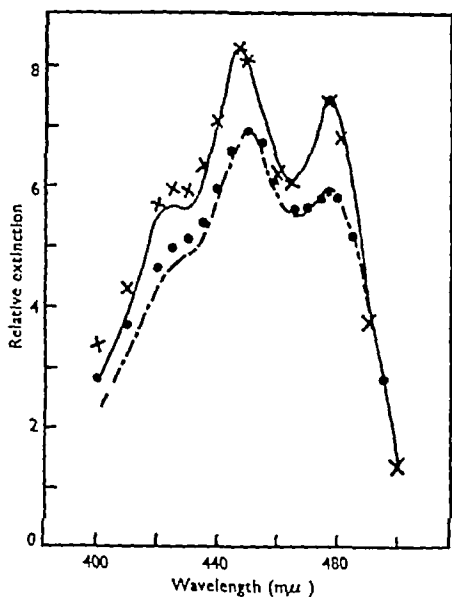


Fig 2 Comparison of visible absorption spectra of pure carotenoids and frog carotenoid extracts —, lutein in ethanol, x x x, frog xanthophyll in ethanol, - - -, β-carotene in n hexane, . . ., frog carotene in n hexane

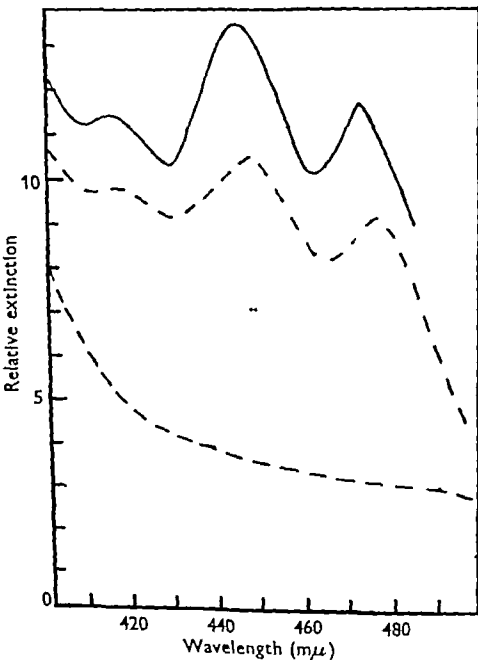


Fig 3 Visible absorption spectra of frog eye extracts —, whole extract (light petroleum 40–60°), - - -, total 'xanthophyll' (acetone), . . ., corrected 'xanthophyll', - - - - -, irrelevant absorption

absorption by the general method of Morton & Stubbs (1946) on the basis of the extent of distortion of the mean 'xanthophyll' curve (see Fig 2) of extracts of other organs. The relative absorptions at the three wavelengths 448 5, 463 and 476 mμ are $E_1=1, 0\ 724, 0\ 881$ respectively, and $E_{448\ 5\ m\mu}(\text{corr})=E_1\times 2\ 216-E_2\times 4\ 688+E_3\times 2\ 472$, where E_1, E_2 and E_3 are observed values at the three wavelengths, respectively.

It was sometimes necessary to examine solutions so dilute that the photoelectric photometer was perforce used outside the recommended range of extinctions. The instrument was therefore calibrated over the range $E=0\ 01$ to $E=1\ 0$ using carotene in light petroleum and 'xanthophyll' esters in acetone or light petroleum. When readings below $E=0\ 2$ were obtained with frog extracts, corrections derived from the calibration curves were applied. In the cases of mono-esterified and free 'xanthophyll' fractions in acetone similar calibration demonstrated that no such correction was necessary. The data in Table 1 were used as a basis for calculations.

Table 1 Spectrophotometric data on carotenoids, used in the analytical work

Substance	λ_{max} (mμ.)	$E_{1\text{ cm}}^{1\%}$	Solvent	Remarks
β Carotene	449 5	2550	Light petroleum	(a)
Lutein	446 5	2550	Ethanol	(b)
Vitamin A	328	1700	CycloHexane	(a)
Frog 'xanthophyll'	448 5	2640	Acetone	(c)
Frog 'xanthophyll' di esters	448	2550	Acetone	(c)
Frog 'xanthophyll' mono-esters	448	2600	Acetone	(c)

(a) Determinations in this laboratory, (b) Zscheile White, Beadle & Roach (1942), (c) values determined in the course of the present work relative to $E_{1\text{ cm}}^{1\%}$ 2550 for pure free lutein.

If, in determining the 'total carotenoid' per frog in a given type of tissue, x frogs were used to provide an extract in y ml and the solution had to be diluted by a factor z so as to bring E_{max} for a 1 cm layer to a convenient figure, then

$$\frac{Ex}{100x/y} = \frac{Eyz}{100z} = Q$$

The use of Q avoids the difficulty that 'total carotenoid' is a mixture of components in which neither λ_{max} nor $E_{1\text{ cm}}^{1\%}$ is necessarily exactly the same. Since, however, $E_{1\text{ cm}}^{1\%}$ at the visible maximum is of the order 2500, i.e. E is about 2 5 for a 1 cm layer of solution of concentration 1 mg/100 ml., $Eyz/250z$ is the weight of solute per frog in mg, so that roughly $0\ 4Q=\text{mg total carotenoid/frog}$.

In this investigation the number of analyses was necessarily very large and the preparation of material time consuming. In choosing methods the aim was the greatest accuracy compatible with speed. It was found to be essential to complete the tests on livers, kidneys and eyes, so far as vitamin A was concerned, in one working day, standing overnight might have caused a loss of as much as 40% of the vitamin. The intact organs could, however, be kept at 0° without appreciable loss (Table 2). The method of decomposing the tissue by boiling with ethanolic KOH and testing the non saponifiable extract was tried side by side

with the procedure already described, and was found to be attended by significant losses or isomerization of carotenoids (Table 3)

Table 2 *Stability of carotenoids in frog tissues stored at 0° after dissection*

Tissue	Period of storage (days)	Total carotenoid $E_{1\text{cm}}^{1\%}$ *	Total vitamin A $E_{1\text{cm}}^{1\%}$ (corr) *
Liver	0	0.0976	0.117
	1	0.0998	0.115
	3	0.0968	0.113
Kidney	0	Not tested	0.543
	1	Not tested	0.557
Muscle	0	0.00487	Nil
	3	0.00482	Nil

* Calculated on wet wt. of tissue

Table 3 *Comparison of analyses for carotenoids and vitamin A carried out on (a) ether extractable lipid and (b) non saponifiable ether extract after treating tissue with ethanolic KOH*

Tissue	Total carotenoid $E_{1\text{cm}}^{1\%}$		Vitamin A $E_{1\text{cm}}^{1\%}$ (corr) *	
	(a)	(b)	(a)	(b)
Cock liver	0.008	0.0072	Not measured	
Horse liver	0.0192	0.0179	0.248	0.263
Hare liver	Negligibly small		0.034	0.035
Frog skin	0.031	0.030	Negligibly small	
Frog liver	0.080	0.089	0.105	0.107
Frog liver	0.227	0.175	0.278	0.243
Frog testes	0.053	0.046	Negligibly small	

* Calculated by the procedure of Morton & Stubbs (1946)

Samples of frog carotenoid fractions with accompanying lipids were used to ascertain the magnitude of manipulative losses in the chromatographic procedures. Over the relevant range, recoveries of 97–99% were obtained. Similar tests with free and combined vitamin A provided equally good recoveries.

The magnitude of the correction for irrelevant absorption at 328 m μ in tests on liver extracts is illustrated in Fig. 4, and on extracts from eyes and kidneys in Fig. 5. The full lines in Fig. 4 show irrelevant absorption at its greatest in the present series (in all there were three such cases), the broken line curves illustrate the normal case in which the irrelevant contribution is about 20% (thirty cases). The carotenoid contribution amounted at 328 m μ to only 3–4% of the total absorption. The need for correction at 328 m μ is much more obvious in the extracts from eyes and kidneys, but the validity of corrected estimates for vitamin A content was confirmed using the SbCl_5 colour test.

When the ultraviolet absorption of an extract suggested the absence of vitamin A, the colour test was used to provide confirmation.

RESULTS AND DISCUSSION

The experimental findings are recorded in tables and figures.

Lipochromes and vitamin A in tadpoles and young frogs

Presence of chlorophyll The absorption spectra of chromatographic fractions containing 'xanthophylls' show some chlorophyll to be present also.

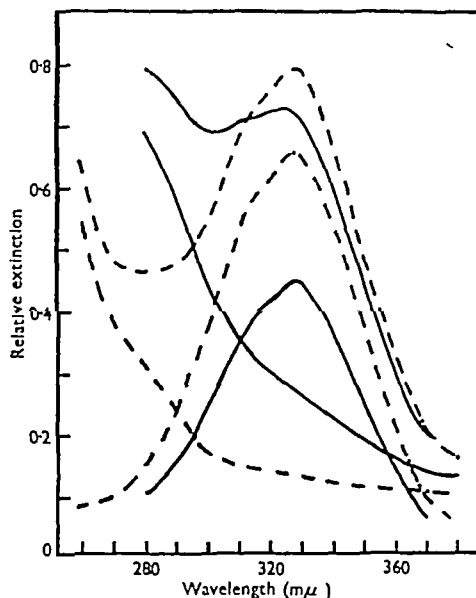


Fig. 4 Ultraviolet absorption spectra of liver extracts illustrating the magnitude of the correction — — —, typical spectrum, ———, atypical spectrum. In each set the top curve is observed, the middle curve corrected and the lowest curve represents irrelevant absorption.

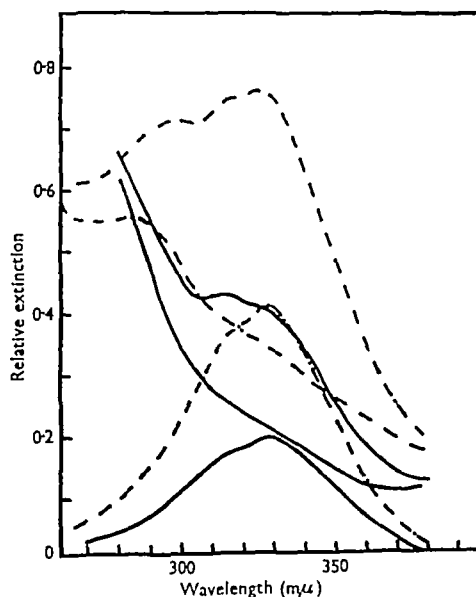


Fig. 5 Typical ultraviolet absorption spectra of eye (— — —) and kidney (——) extracts, showing gross, corrected and irrelevant absorption.

Thus, tadpole extracts exhibit maxima at 662-664, 615 and 432-434 $m\mu$, $E_{662\text{ }m\mu}/E_{615\text{ }m\mu}=4.2$ Harris & Zscheile (1943) give for chlorophyll *a* λ_{max} 661.5, 615.5 and 431.5 $m\mu$, $E_{661.5\text{ }m\mu}/E_{615.5\text{ }m\mu}=6.3$ The low value for the ratio of the intensities of the two chlorophyll bands (Figs 6 and 7) suggests the presence of chlorophyll *b*. By applying the method of Comar & Zscheile (1942) to a

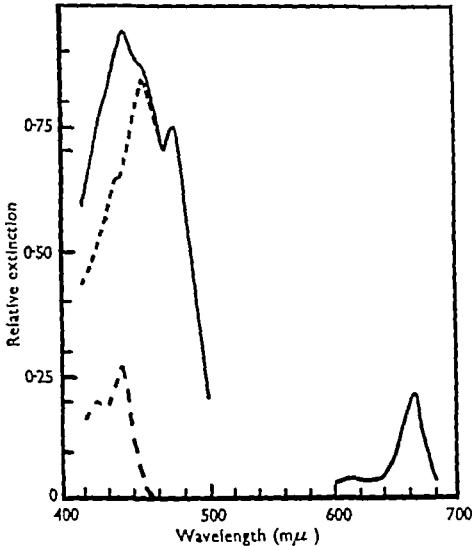


Fig 6 Visible absorption spectra of frog tadpole 'xanthophyll' in acetone —, 'xanthophyll' from chromatogram, - - -, computed chlorophyll contribution, . . ., xanthophyll contribution.

fresh ether extract of tadpoles, chlorophyll *a* was found to preponderate over chlorophyll-*b* by a factor of 7.5 to 1. The observed absorption in the region 400-500 $m\mu$ is therefore a summation of contributions from 'xanthophyll' and 'chlorophyll'. The absorption in the region 600-700 $m\mu$ is due solely to the latter, and from the absorption curve for pure chlorophyll-*a*, the curve from the red region can be continued through to the violet using the following relative *E* values

λ	662	400	412	420	430
<i>E</i>	0.976	0.7	0.913	0.875	1.218
λ	432	440	450	460 $m\mu$	
<i>E</i>	1.225	0.595	0.074	0.018	

The 'xanthophyll' contribution in tadpole extracts is then obtained by difference (Fig 6). In two samples, the 'chlorophyll' and 'xanthophyll' were separated by chromatography on weakened alumina and the validity of the difference method confirmed.

In extracts of natterjack tadpoles the chlorophyll contribution was computed in a similar manner, but an additional maximum near 420 $m\mu$ due to the presence of an artefact was observed when the

'xanthophyll' contribution was obtained by difference. This substance, which showed a maximum at 412 $m\mu$, was probably a degradation product of chlorophyll, it was easily removed by adsorption on alumina and the anomalous 'xanthophyll' absorption was corrected by subtraction (Fig 7). Carotenoids are decomposed by exposure to light in the presence of chlorophyll and oxygen (Pepkowitz, 1944), it is necessary, therefore, to protect the

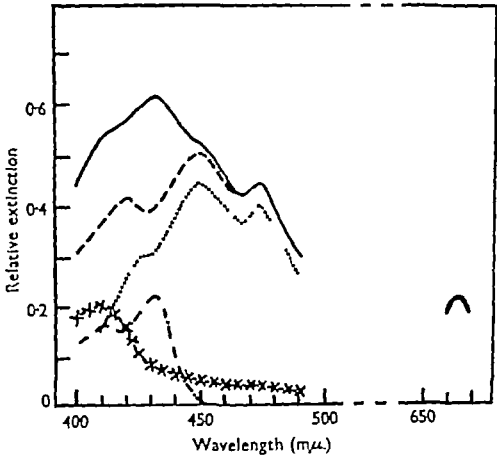


Fig 7 Visible absorption spectra of natterjack tadpole 'xanthophyll' in acetone —, 'xanthophyll' from chromatogram, - - -, computed chlorophyll contribution, . . ., absorption of 'xanthophyll' plus artefact, x-x-x, artefact contribution, —, 'xanthophyll' contribution.

extracts as much as possible. Vitamin A in traces is very difficult to detect in such mixtures, and the only way in which its presence was demonstrated in natterjack tadpoles was by the antimony trichloride colour test applied to extracts from the dissected eyes.

Occurrence of carotenoids. The number of tadpoles used was ample for the methods applied, and the data obtained are shown in Tables 4-6. The tadpoles were grouped in accordance with the stages of development A-H which can be fitted into an approximate time scale (see Fig 8).

Comment. The chlorophyll is almost certainly all present in the digestive tract. The tadpoles were kept for 3 days in captivity after collection, but although they were active and faecal excretion copious, it was found that those collected at stage A had to be kept for 9 days before chlorophyll disappeared. In tadpoles caught at different ages the chlorophyll content is considerable in stages B, C and D, less in E, very low in F and negligible in G. This suggests a decrease in food intake which is borne out by the weights and the carotenoid analyses (Table 4).

Table 4 *Carotenoids in tadpoles of Rana temporaria at various stages of development*

(Chlorophyll was present in A-F, not detected in G and H)

Stage	Date and number used (in brackets)	Average wt (g)	Carotene/tadpole (μg)	'Xanthophyll'/tadpole (μg)	Ratio 'xanthophyll'/carotene
A, young tadpoles, 10-20 mm	2 v 47 (312)	0.049	0.012	0.11	9.7
B, tadpoles, 20-30 mm	2 v 47 (236)	0.12	0.033	0.30	9.1
C, tadpoles, 30-40 mm, rear leg just seen	20 v 47 (75)	0.32	0.31	1.78	5.6
D, rear legs less than 4 mm	4 v 1 47 (82)	0.22	0.27	0.03	3.4
E, rear legs greater than 4 mm	4 v 1 47 (52)	0.25	0.40	1.21	3.0
F, fore limbs formed, some free	4 v 1 47 (73)	0.22	0.25	0.92	3.7
G, all four limbs well formed, long tails persisting	10 v 1 47 (88)	0.19	0.23	0.72	3.1
H, small frogs, tails almost resorbed	10 v 1 47 (93)	0.15	0.25	0.76	2.8

Table 5 *Rate of disappearance of carotene and 'xanthophyll' during metamorphosis of tadpoles*

	Average amounts of pigments (μg /tadpole)		Loss (%)
	Stages C, D and E*	Stages F, G and H†	
Carotene	0.33	0.25	24
'Xanthophyll'	1.31	0.80	38

* Feeding freely

† Fasting

Table 6 *Rate of disappearance of carotene and 'xanthophyll' from young tadpoles caught at stage A and kept in captivity without food*

	Pigments expressed as (μg /tadpole)		Loss (%) (approx)
	Tested 3 days after capture	Tested 9 days after capture*	
Carotene	0.0116	0.0093	20
'Xanthophyll'	0.112	0.087	22

* Chlorophyll absent

The amount of carotenoid in the original eggs is less than one quarter of that present in active tadpoles. Now the ratio of 'xanthophylls' to carotenes in the plant world, although variable, is only very rarely outside the range 3-10. In young tadpoles the ratio is initially about 9 and falls to about 3 at the time of metamorphosis and then falls further in young frogs. Up to maximum growth 'xanthophyll' storage increases more rapidly than carotene storage. The abrupt fall in 'xanthophyll' level when the rear legs first appeared obviously needs confirmation, but the very small concomitant drop in carotene affords a hint that it is not illusory. The fact that little or no food is eaten during metamorphosis allows the disappearance of carotenoids to be followed. The data given in Fig 8 and Tables 5 and 6 suggest that the main process does not discriminate sharply between carotene and 'xanthophyll'. The change in the 'xanthophyll'/carotene ratio recorded in Table 4 is not then simply a matter of selective

'utilization' as measured by rate of disappearance. If, during the period of metamorphosis, carotene had been used for the synthesis of vitamin A, the hydrocarbon might have been expected to disappear more quickly than the 'xanthophyll' (mainly lutein in the tadpole). There is no doubt that vitamin A is formed in the tadpole. Natterjack tadpoles each contained about 0.027 μg in the eyes and newly metamorphosed frogs about 0.1 μg .

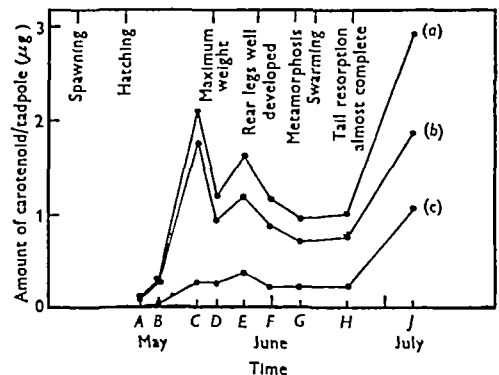


Fig 8 Carotenoids in the developing tadpole (a) total carotenoid, (b) total 'xanthophyll', (c) carotene. The stages A-H are specified in Table 4

It is now clear that the main, if not the sole site of conversion of carotene to vitamin A in mammals is the absorptive portion of the gut, and it seems very probable (although obviously difficult to prove) that the same holds true for the tadpole. It is very unlikely that the 'xanthophyll'/carotene ratio in the kind of vegetation ingested is ever as high as 9, the appearance of this ratio in the early stages of development (A and B) suggests a selective utilization of carotene in the gut wall. On this hypothesis, as development proceeds and food intake increases, the 'carotenase' enzyme system becomes unable to cope with more than a fraction of the carotene presented to it. The residue will follow the normal pathway of lipid absorption, and the 'xanthophyll'/carotene

ratio will fall until it reaches a figure much nearer to that of the diet. The carnivorous young frogs will continue to convert a proportion of ingested carotene and to absorb carotene and 'xanthophyll' non selectively.

It is possible that in the future the hatching of frog spawn and the growth of tadpoles in captivity on artificial diets may be of service if either carotene or 'xanthophyll', or both, can be excluded.

Lipochromes and vitamin A in adult frogs

Vitamin A There does not appear to be any pre-formed vitamin A in the diet of the frog. The vitamin formed *in vivo* from provitamin is found in the liver (about 85%) and in the eyes and kidneys in approximately equal amounts (Tables 9 and 11). In each site it occurs mainly as ester. The amount of vitamin A in kidney (about 30 $\mu\text{g/g}$) is very much larger than that found in mammalian kidneys.

Carotenoid distribution Carotene as such is less efficiently absorbed than the hydroxylated carotenoids since the 'xanthophyll'/carotene ratios are approx 3:1 in the diet, 2:1 in the faeces and, in the body, 4:7:1 for females and 5:7:1 for males (October 1947).

In contrast with the restricted distribution of vitamin A, carotenoids occur in all the organs studied (Table 9). They also appear to occur in nerve tissue and in glands such as the thymus. If the amounts of carotenoids at different sites are expressed in terms of that present in the skin (chosen for comparison because of its relative constancy), the results in Table 7 give an idea of the approximate distribution over the body. In males, the skin, liver and muscle are important, in the female the gonads dominate the situation (Table 7).

Table 7 *Relative amounts of total carotenoid in frog organs*

(These are mostly seasonal averages, without distinction between the sexes)

Skin*	1
Ovaries and mature eggs	2.65
Liver	0.7
Muscle (total leg)†	0.17
Oviducts	0.12
Immature ovaries	0.1
Stomach	0.05
Testes	0.035
Small intestine, fat bodies, kidneys	0.23-0.28
Oesophagus	0.16
Tongue	0.09
Pancreas, large intestine, lungs, eyes, heart	0.004-0.008
Seminal vesicles, spleen	Traces

* Skin carotenoid is taken as unity, because the absolute amount is large and is less influenced by sex and season than other tissues rich in carotenoids.

† Carotenoids of leg muscle/ventral and side muscle/arm muscle = 36/2/1

Table 8 *Weights of frog organs expressed as percentage of body weight*

Organ	Minimum	Maximum
Testes	0.32	3.66 (Aug)
Ovaries	0.925 (Apr)	36.0 (Mar)
Oviducts	1.47 (Apr)	17.5 (Dec)
Livers (male)	1.4 (Apr)	3.6 (Nov-Dec)
	1.3 (Apr)	2.3 (Oct-Dec)
Fat bodies (male)	Nearly 0 (Apr)	8 (Aug-Sept)
	Nearly 0 (Apr)	7 (Aug-Sept)
Skin	Approx 10	
Leg muscle	Approx 15	
Kidneys	0.3-0.5	
Pancreas	0.06-0.13	
Stomach	Approx 1.0	
Lungs (male)	0.42	
	0.25	
Tongue	0.8	
Eyes	Approx 1.0	
Heart	Approx 0.25	

Although the left fat body is larger than the right one, the carotenoid content per unit weight is the same. The adrenals are somewhat richer in carotenoids than the kidneys. In all tissues α -carotene seems to be practically absent and the 'xanthophyll' is nearly all lutein. Very small amounts of other carotenoids were sometimes found, but there was too little for characterization. A few toads (*Bufo vulgaris* and *B. calamita*) have been studied in the course of the present work and no significant differences were observed from experience on frogs.

One of the implications of the distribution data is that the carotenoids are not merely distributed with the fat, thus for females in October 1947 the concentrations of carotenoids in fat varied from about 0.01 mg/g for fat bodies to 8.4 mg/g for skin, with liver 2.7 mg/g and ovaries 1.1 mg/g (Table 15). The external appearance of frogs and toads owes little or nothing to the lipochromes present in the skin. The outer layers are rich in melanins and in pigments soluble in dilute ammonia.

Lederer & Rathmann (1938) found small proportions of vitamin A₂ in *R. esculenta* liver fat, but in the present work with *R. temporaria* no A₂ was observed, confirming the finding of Gillam (1938).

Ultraviolet absorption spectra of lipid fractions from frog organs

The absorption spectra of liver extracts have already been referred to (Fig. 4). Observations on extracts from other organs are recorded. Selective absorption of undetermined origin in the region 280-300 m μ was recorded in lipids from the eyes of frogs and toad (Fig. 5) and also rather erratically in frog kidneys. With one exception (November 1946) the testes extracts showed continuous end absorption over the range 250-400 m μ . The aberrant sample showed an inflexion near 265 m μ .

Extracts from ovaries were more interesting. Material from the mature ovaries of frogs in the pre-hibernation period exhibited the well resolved absorption shown in Fig. 9. The characteristic curve

Table 9 *Distribution of fat, carotenoid and vitamin A in the organs of sixty male frogs, divided into three main groups*

	Group	Mean body length (cm)	Mean weight (g)			
	<i>A</i>	6.6	22.8			
	<i>B</i>	5.6	14.2			
	<i>C</i>	5.1	11.0			
In table below means were not measured, a dash (—) means 'not measured'						
Organ	Wt as % body wt	Wt of fat (mg /frog)	Total carotenoid <i>Q</i> × 10 *	Carotene (μg /frog)	'Xanthophyll' (μg /frog)	Vitamin A (μg /frog)
Fat bodies						
<i>A</i>	0.058	5.6	0.33	0.158	1.40	0
<i>B</i>	0.061	5.4	0.08	—	—	0
<i>C</i>	0.030	2.5	0.02	—	—	0
Pancreas						
<i>A</i>	0.117	—	0.11	—	—	0
<i>B</i>	0.105	—	0.06	—	—	0
<i>C</i>	0.120	—	0.057	—	—	0
Kidneys						
<i>A</i>	0.35	—	0.29	—	—	5.3
<i>B</i>	0.35	—	0.235	—	—	2.4
<i>C</i>	0.35	—	0.18	—	—	1.6
Testes						
<i>A</i>	1.20	4.1	0.33	0.59	0.77	0
<i>B</i>	1.24	2.7	0.22	0.38	0.56	0
<i>C</i>	1.23	1.7	0.17	0.32	0.40	0
Eyes						
<i>A</i>	0.86	—	—	—	—	3.0
<i>B</i>	1.02	—	—	—	—	2.3
<i>C</i>	1.30	—	—	—	—	2.1
Liver						
<i>A</i>	2.31	3.6	4.46	3.4	13.0	39.6
<i>B</i>	2.31	2.9	3.84	3.3	11.5	20.6
<i>C</i>	1.92	2.2	2.32	1.25	7.2	12.1
Tongue						
<i>A</i>	0.63	—	0.11	—	—	0
<i>B</i>	0.74	—	0.082	—	—	0
<i>C</i>	0.72	—	0.078	—	—	0
Stomach						
<i>A</i>	1.09	—	0.48	0.52	1.24	0
<i>B</i>	1.12	—	0.44	0.49	1.16	0
<i>C</i>	1.27	—	0.34	0.35	0.90	0
Skin						
<i>A</i>	11.6	16.2	12.5	7.25	43.6	0
<i>B</i>	11.1	12.6	8.5	5.02	27.8	0
<i>C</i>	11.1	9.7	6.9	2.19	24.4	0
Leg muscle						
<i>A</i>	17.3	18.3	3.42	1.04	10.6	0
<i>B</i>	15.6	10.6	2.04	0.97	6.9	0
<i>C</i>	14.5	6.8	1.54	0.69	5.0	0

* See p. 615 of text

with maxima at 292.5, 281.5, 271 and 261 $m\mu$ was reproduced in the non saponifiable fraction. The spectrum is qualitatively indistinguishable from that shown by provitamins D, e.g. 7 dehydrocholesterol and ergosterol. Without using a large amount of material it is not possible to decide which of the several substances known to exhibit this spectrum occurs in the frog ovaries. Applying a

correction procedure based on the method of Morton & Stubbs (1946) using reference data obtained on highly purified ergosterol (which is spectroscopically almost identical with 7 dehydrocholesterol) the corrected $E_{1\text{ cm}}^{1\%}$ 281.5 $m\mu$ value was 0.117, corresponding with a concentration of 1 in 2430 in the ovaries [$E_{1\text{ cm}}^{1\%}$ (ergosterol) = 285]. A calculation based on the work of Boyd (1938) indicates

that mature ovaries contain about 0.8% of cholesterol. It thus appears that the ovarian steroids contain approx. 0.5% of 7-dehydrosteroid.

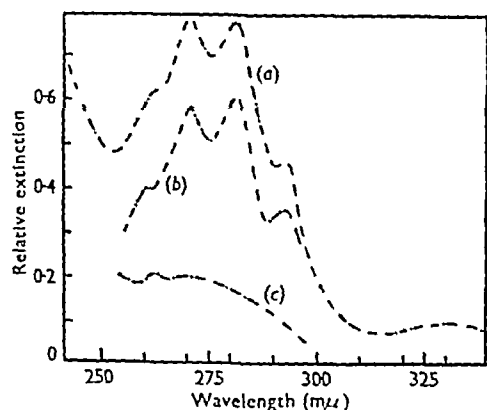


Fig. 9 Ultraviolet absorption spectra of the extract of mature frog ova in ethanol: (a) gross absorption, (b) corrected curve, (c) irrelevant absorption.

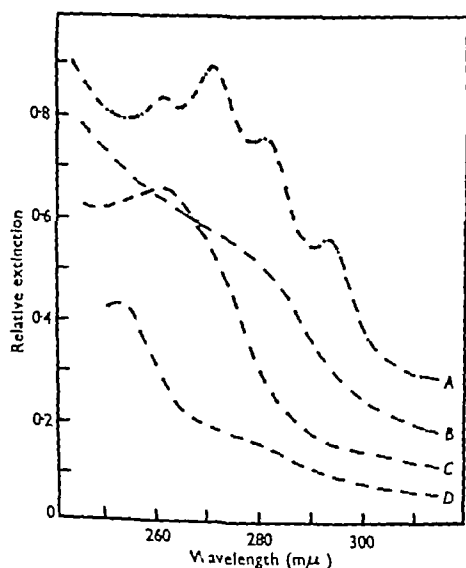


Fig. 10 Ultraviolet absorption spectra of frog ova extracts: A, mature ova just prior to oviposition (ethanol), B, December ova immature through deprivation (ethanol), C, immature ova from frogs which yielded mature ova (curve A) (in ethanol), and D, immature ova from spring frogs 1947 (cyclohexane).

The characteristic spectrum only appears when the ovaries are mature, but it persists through the period of hibernation and is seen (Fig. 10A) just before oviposition occurs. There is some loss of definition and possibly a decrease in intensity, but it is difficult to say how much of the apparent loss is fictitious, since the protein layer and water are

accumulated at this time. Curve C (Fig. 10) shows the absorption spectrum of an extract of immature ovaries obtained from the same frogs as the ripe ova in March 1948. Curve D refers to immature ovaries obtained in the spring of 1947 and B to ovaries from frogs which had fasted for a long time (p. 625). The weak selective absorption at 330 mμ (Fig. 9) is not due to vitamin A or carotenoids.

The data on extracts from other organs show no well defined spectra, but there are numerous indications of the presence of traces of absorbing constituents. Much larger amounts of material would have to be used in following up these observations.

The occurrence of the ergosterol like bands in mature ovaries is surprising. It suggests that the hypothesis of a role for 7-dehydrosteroids other than that of a provitamin D is worthy of study.

Seasonal variations in carotenoids and vitamin A

A considerable body of metrical data accumulated during the study of some 600 frogs, the main features of which are summarized in Tables 8 and 10. The quantitative picture which emerges from a study extending over only two seasons is in some respects rather tentative, but it is likely to be broadly correct. In 1946 the liver fat for both sexes showed a 'xanthophyll'/carotene ratio (X/C) of 1 in the autumn, but a year later X/C was about 3 (Table 12). The

Table 10 Size and weight of frogs used at different dates in the present work.

(The average body lengths were computed from the data of March (1937), except those marked with an asterisk.)

Date of dissection	Number		Average body wt (g)	Average body length (cm)
	Males	Females		
14 viii. 46	—	18	27.5	6.9
19 viii. 46	18	—	20.4	6.5
9 ix. 46	20	—	30.5	7.3
11 ix. 46	—	19	25.6	6.9
17 x. 46	18	—	26.8	6.9
21 x. 46	—	18	26.7	6.5
6 xi. 46	20	—	29.3	7.1
12 xi. 46	—	20	34.5	6.9
9 xii. 46	19	—	25.4	6.7
16 xii. 46	—	21	31.1	6.7
1 iv. 47	—	13	29.5	6.8
7 iv. 47	18	—	22.7	6.2
13 iv. 47	—	14	14.5	6.5
12 v. 47	20	—	20.3	6.5
28 v. 47	—	19	16.3	6.3
8 vii. 47	14	—	25.3	7.1
22 vii. 47	—	19	19.9	6.7
3 x. 47	17	—	13.1	5.5*
8 x. 47	—	17	16.8	5.9*
30 x. 47	20	—	22.8	6.6*
30 x. 47	20	—	14.2	5.6*
30 x. 47	20	—	11.0	5.1*

weather remained mild until early December in 1946 and the frog fat bodies were much larger in October and November than a year later. Apart from complications arising from the fact that no two years have the same weather conditions,

the age (size) variable must be distinguished from the variable of an annual cycle in the frog at all ages after puberty

A group of sixty male frogs, captured near Norwich in October 1947, was divided into three groups. Table 9 summarizes the experimental data. The weights of organs expressed as percentage of body weight are reasonably constant apart from the cases of fat bodies and eyes. No simple expression can be found to cover the lipochrome or vitamin A variations, but both increase with increasing age.

An experiment with a further group of sixty male frogs caught in the spring of 1948 at Hillside showed that two groups of twenty not differing in average size (6.7 cm) differed little in respect of skin carotenoid, liver carotenoid and liver vitamin A. A third group of the same size showed very much less liver vitamin A and slightly less liver carotenoid. The frogs in the third group, unlike the others, were spent (seminal vesicles empty, and testes shrunken), and, as will be seen, it is plausible to connect that fact with the decrease. Only rather gross signs of seasonal variations, reinforced by data on the average body length of the frogs employed (see Table 10), can therefore be regarded as significant at this stage in the problem.

The variations in carotenoid and vitamin A content of certain organs during the annual cycle are illustrated in Figs 11-15. The results obtained on the first sixty male frogs in groups A, B, C (p. 620) have been taken into account in the discussion.

Vitamin A

(1) *Liver* Males and females showed a summer maximum with a fall in early autumn and a rise in late autumn (Fig. 11). There may have been a real decrease prior to

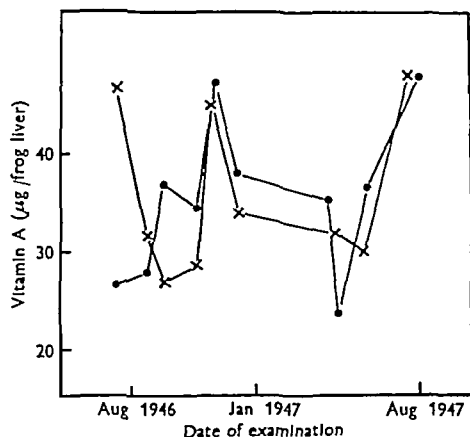


Fig. 11 Seasonal variation in frog liver vitamin A
x-x-x, male, ---, female

hibernation, but there was no sign of a drop during winter. Some females shed their eggs in the laboratory without pairing (April 1947) and showed low liver levels afterwards. Similarly, in 1948, the three sets of males already referred to showed respectively 36.1, 36.2 and 20.7 µg/liver, the low value being obtained in the 'spent' animals.

(ii) *Eyes* Both sexes showed a late autumn maximum and a fall in vitamin A before hibernation. Males but not females appeared to exhibit a winter minimum (Fig. 12).

(iii) *Kidney* Beyond the fact that kidney storage of vitamin A seems to be maximal in autumn, there is too little evidence for postulating a true annual cycle (Fig. 12). There is little evidence that hibernating frogs deplete their vitamin A reserves, but reproduction causes a heavy drain. The temperature coefficient of the enzyme system responsible for the conversion of provitamin A to vitamin A is probably more responsible for the summer rise in the liver reserve than the copious feeding.

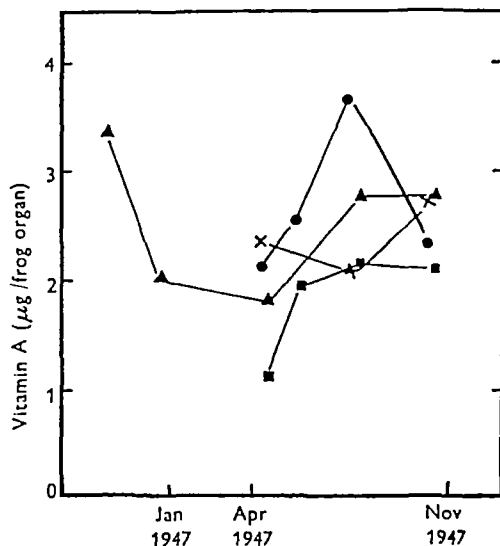


Fig. 12 Seasonal variation in frog eye and kidney vitamin A: x, eye (male), ●, kidney (male), ▲, eye (female), ■, kidney (female)

Table 11 shows for frogs at different seasons the vitamin A contents of the three organs expressed as percentage of the total reserves. The seasonal variations suggest sex differences, and show clearly that the liver is always the main storage organ. So far as the data go, 75-88% of the vitamin A occurs as ester in all sites with 80% as a typical figure. A few discrepant observations were, however, made.

Table 11 Seasonal variations in the distribution of vitamin A in frogs of both sexes

Season	Sex	Average body length (cm)	Vitamin (% of total)		
			Liver	Eyes	Kidneys
Spring	M	6.2	87.0	6.9	6.1
	F	6.5	88.4	7.2	4.5
Summer	M	7.1	88.8	4.1	7.1
	F	6.7	90.2	5.5	4.3
Autumn	M	5.5	77.5	11.7	10.9
	F	6.0	82.7	9.9	7.5

Total carotenoid

(i) *Liver* No reduction was manifested during hibernation for either sex, in fact there may have been a small rise for males. In both males and females the decrease on mating was followed by a rise to a summer maximum. There was

then a gradual decrease continuing until December with nothing analogous to the autumnal rise in hepatic vitamin A (Fig 13)

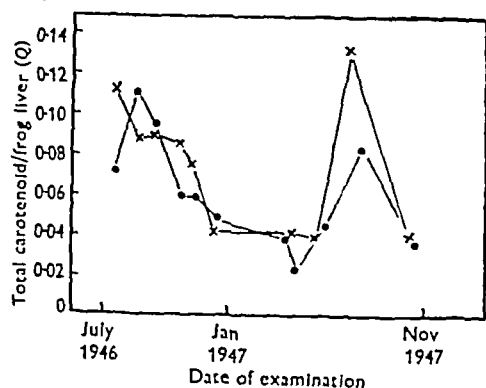


Fig 13 Seasonal variation in frog liver total carotenoid x-x, male, ●-●, female (Q, see p 615)

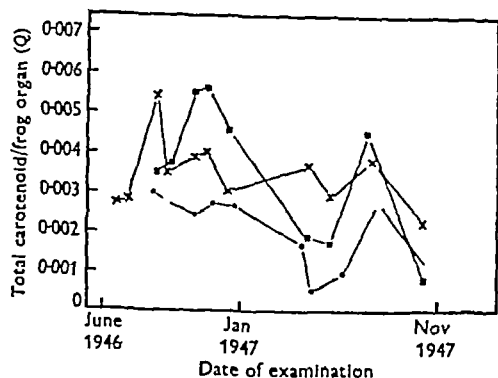


Fig 14 Seasonal variations in testes and fat body total carotenoid x-x, testes, ●-●, female fat body, ■-■, male fat body (Q, see p 615)

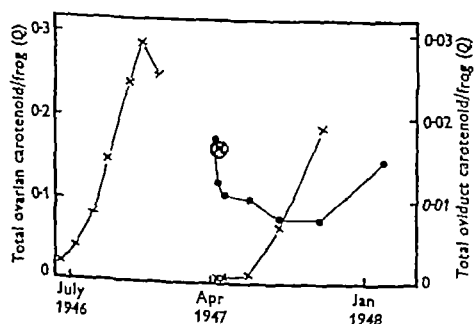


Fig 15 Seasonal variation in ovarian and oviduct total carotenoid x-x, ovaries, ●-●, oviducts. The ringed cross represents the total carotenoid content of mature ova just prior to oviposition (Q, see p 615)

(u) *Sex organs* (Figs 14 and 15) The gonads and also the fat bodies showed marked seasonal variations in carotenoid content. The testes were very poor in lipochrome after mating and remained so until August when a sharp rise occurred, there was some fluctuation from September to

November, but no clear cut secondary maximum was recorded. Transfer to the testes seems to occur during or just after hibernation. Immature ovaries contain very little carotenoid, but just prior to hibernation a thirtyfold rise had been attained. Whether or not there is a drain on ovarian carotenoids during the winter sleep cannot be stated, as contradictory results were obtained in the 1947 and 1948 seasons, but it could not be large. The oviducts showed a rise in late autumn, but there was no drop in winter (when the ducts are functioning actively). On spawning, the ducts atrophy and there is a sudden decrease to a low level which is maintained through the summer.

Male fat bodies showed a marked rise in November, whereas in females the levels were lower and fairly constant throughout the autumn. Hibernation losses occurred in both sexes and for females especially, marked depletion occurred on spawning with recovery during summer feeding to lower levels for females than for males.

(u) *Kidneys, skin and organs of the digestive tract* No significant seasonal variations in kidney or skin carotenoids could be observed for either sex. Some variations occurred in the digestive tract. Males showed summer and autumn peaks in the stomach, but females only showed the autumn rise. In the small intestine there was a maximum in autumn for both sexes. During winter the intestinal carotenoids were maintained (males) or even rose a little (females).

(iv) *Leg muscle*. A temporary rise appeared to occur in the autumn.

(v) *Tongue, lungs, pancreas, eyes* There was evidence of an autumn maximum, but the levels were fairly steady on the whole. In the male pancreas an increase is suggested during or immediately after hibernation, but in the female there is a fall on egg laying. In the male eye the total 'xanthophyll' drops in early summer and then rises, but there is no clear cut autumn maximum in the female. Carotene was found in traces ($< 0.05 \mu\text{g/eye}$) but 'xanthophyll' preponderates in the eye.

Comment on seasonal variations

The foregoing results are not easy to interpret fully. A tendency for carotenoid content to rise after heavy feeding is quite definite, but some organs respond quickly and others much more slowly. Furthermore, there may be a seasonal change in storage sites. For example, during the early summer feeding flush, the liver lipochrome increases markedly, yet the muscle content does not rise appreciably, during the autumn feeding this is reversed. However, it is not possible to distinguish between carotenoids carried with fat in lipid transfer and carotenoids mobilized in accordance with the cycle of specific utilization if such exists.

The gonadal cycle in the female results in a larger storage of carotenoids in the developing ovaries than in the whole of the rest of the body. Expulsion of ova is by far the largest cause of carotenoid loss, probably larger even than the total oxidative degradation. The cycle in the testes and oviducts is not responsive to dietary changes, but this is no more than a hint at functional significance. The sex difference in carotenoid storage in the fat bodies recalls the dissimilar gonadal sequence, for spermatogenesis is spread over

no more than six weeks, whereas ovarian development proceeds steadily until the autumn acceleration of growth

Although muscle and kidney stores fluctuate relatively little it is noticeable that the absolute amount stored in muscle (especially for males) is quite appreciable. Muscle fat and carotenoid is probably mobilized readily. It is interesting that, although frogs feed soon after mating, carotenoid storage does not occur until late May. There is a similar lag in liver glycogen and blood sugar increment (Smith, 1947)

The 'xanthophyll'/carotene ratio (X/C)

The relative amounts of 'xanthophyll' and carotene stored differ markedly in the various organs. For frogs of different sizes the quotient varies as follows: testes 1.2-1.5, liver 3.5-5.8 and stomach 2.4-2.55, skin 5.5-11, muscle 7-10. There is, however, no uniform trend with increasing age in different organs. The data on seasonal changes are summarized in Table 12. It is quite possible that the picture is distorted by some local or other factor connected with the availability of particular kinds of food, in fact marked annual differences have been observed. Some of the grosser effects can, however, be interpreted. When carotene predominates as it

appears to do in female livers (August-December 1946), transfer of 'xanthophyll' from liver to gonads is the probable explanation, because the quantitative demand of female gonads for 'xanthophyll' is great. During spermatogenesis (when the total carotenoid is maximal) the X/C ratio for testes is doubled, but the reason is not clear.

Free and combined 'xanthophyll'

Seasonal effects Table 13 shows the distribution of free 'xanthophyll', and mono- and di-esterified 'xanthophyll' as a percentage of the total. Although high accuracy cannot be claimed for the figures it is obvious that the organs fall into two classes in which the 'xanthophyll' is respectively mainly free and mainly combined. The significance of the data in Table 13 is not clear, because it implies a distribution of esterases for which no explanation can be offered.

Comparison of fat and carotenoid storage

Since lipid is the normal vehicle for carotenoids it is important to know whether the storage and mobilization of lipochrome is correlated with that of fat. Tables 14 and 15 show that it is not. (The ratio fat/carotenoid (C/F) is approximate, since the figures for total carotenoid are obtained by the roughly

Table 12 *Seasonal changes in the 'xanthophyll'/carotene ratio in frog organs*

Organs	Sex	1946						1947				
		July	Aug	Sept	Oct	Nov	Dec	Apr	Apr	May	July	Oct
Testes	—	—	2.5 (?)	1.95	1.06	0.87	1.06	0.85	—	0.88	1.92	1.42
Ovaries	—	7.2	6.3	7.2	7.2	6.7	7.4	7.7	11.6	9.6	11.2	8.2
Fat bodies	M	—	—	7.7	5.7	5.0	4.9	6.7	—	16.0	10.9	7.9
	F	—	—	—	5.0	4.4	—	—	7.2	5.1	9.2	—
Skin	M	—	—	—	—	—	—	5.0	—	4.6	5.3	4.8
	F	—	—	—	—	—	4.1	2.6	4.3	4.1	4.6	2.8
Liver	M	1.12	0.72	1.97	1.59	1.19	1.46	1.51	—	1.57	1.74	3.0
	F	1.02	0.51	0.78	0.88	0.71	0.95	0.81	1.58	1.38	1.74	2.8
Kidney	M	—	—	—	—	—	—	3.6	—	—	5.0	4.5
	F	—	—	—	—	—	—	2.9	—	2.7	9.3	2.2
Leg muscle	M	—	—	—	—	—	—	—	—	—	10	7.5
	F	—	—	—	—	—	—	—	—	—	8.3	8.7
Lungs	M	—	—	—	—	—	—	3.0	—	—	—	—
	F	—	—	—	—	—	—	—	—	—	3.9	9.0
Pancreas	M	—	—	—	—	—	—	6.8	—	—	15.8	7.7
	F	—	—	—	—	—	—	—	—	—	—	6.2
Stomach	M	—	—	—	—	2.1	1.7	1.8	—	2.4	—	2.1
	F	—	—	—	—	—	—	—	—	—	2.4	—
Intestine	M	—	—	—	—	—	4.1	2.6	—	—	—	4.0
	F	—	—	—	—	2.6	—	—	—	—	4.2	3.1
Large intestine	M	—	—	—	—	—	—	1.9	—	—	—	0.9
	F	—	—	—	—	—	—	—	—	—	2.3	1.0
Tongue	M	—	—	—	—	—	—	—	—	—	3.8	1.1
	F	—	—	—	—	—	—	—	—	—	1.8	2.2
Oesophagus	M	—	—	—	—	—	—	—	—	—	—	2.4
	F	—	—	—	—	—	—	—	—	—	4.9	—
Oviducts*	—	—	—	—	—	—	—	0.83	0.67	0.76	0.72	1.0

* Oviducts alone fail to show 'xanthophyll' predominating at any season of the year. X/C ratio was 0.88 in Jan. 1948.

Table 13 Seasonal distribution of free 'xanthophyll' and xanthophyll mono and di esters in frog organs

Organ	Season	Sex	Percentage of total 'xanthophyll' as		
			Di ester	Mono ester	Free
Skin	Summer	F	68	15	27
Skin	Summer	M	61	19	20
Skin	Autumn	M	68	15	17
Skin	Spring	M	74	13	13
Ovaries	Summer	—	52	33	15
Ovaries	Autumn	—	60	27	13
Ova	Spring	—	58	20	22
Liver	Autumn	M	9	18	73
Muscle	Autumn	M	12	8	80
Muscle	Autumn	F	8	8	84
Muscle	Spring	F	10	9	81
Oviduct	Autumn	—	11	22	67
Testes	Winter	—	7	23	70
Fat bodies	Winter	M	11	34	55
Small intestine	Winter	M	6	13	81

Table 14 Total carotenoids and total fat of organs of male frogs of different sizes

(Mean body lengths Group A, 6.6, B, 5.6, C, 5.1 cm Total carotenoid calculated on basis of mean $E_{1\text{ cm}}^{1\%}$ 450 m μ 2500)

Group	A			B			C		
	Fat (mg)	Carotenoid (μ g)	C/F (mg/g)	Fat (mg)	Carotenoid (μ g)	C/F (mg/g)	Fat (mg)	Carotenoid (μ g)	C/F (mg/g)
Testes	4.1	1.3	0.3	2.7	0.87	0.32	1.7	0.68	0.4
Liver	3.6	18	5.0	2.9	15.4	5.3	2.2	9.3	4.2
Skin	16.2	50	3.1	12.6	34	2.7	9.9	28	2.9
Muscle	18.3	13.7	0.75	10.6	8.2	0.77	6.8	6.2	0.9

Table 15 Seasonal variations (1947) in ratio of total carotenoid/fat in frog organs

(Ratios expressed as mg total carotenoid/g fat)

Organ	Sex	April	May	July	October
Testes	—	—	0.63	0.44	0.42
Ovaries	—	3.4	1.3	1.12	0.68
Fat bodies	M	—	0.49	0.01	0.09
	F	—	0.02	0.01	0.12
Kidneys	F	—	0.66	0.3	1.5
	M	—	0.28	0.47	1.4
Liver	F	3.3	3.6	1.6	3.6
	M	3.2	1.6	2.7	1.1
Leg muscle	M	—	—	—	1.8
	F	—	—	0.34	1.2
Skin	M	2.8	3.5	3.1	3.2
	F	3.4	2.3	8.4	4.0
Oviducts		0.3	0.3	0.2	0.4

correct assumption of an average $E_{1\text{ cm}}^{1\%}$ 450 m μ of 2500 for the constituent pigments) Variations with size are obviously less important than inter organ variations Seasonal variations (Table 15) are striking, but again there is no correlation between fat transfer (or metabolism) and the fate of carotenoids

Vitamin A fed to frogs

One male and one female were each given 1 drop (approx 3500 i.u.) of an ester concentrate daily for a month, with no

other food Much of the vitamin was excreted but the liver level rose threefold Some vitamin appeared in the extracts from lungs, fat bodies and testes, but not in the skin or the ovaries The experiment indicates poor assimilation, but this may have been due to the fact that the animals (like all frogs in captivity) refused to eat spontaneously The absence of vitamin A from the ovaries is consistent with the observation that it is not detectable in newly spawned eggs

Fasting frogs

The ability to do without food for long periods is a well known characteristic of frogs In an experiment in which twenty five female frogs were kept without food from 18 July to 9 December 1947, the fat bodies had been exhausted and body weights had declined by some 35% Ovarian development was greatly retarded The decreases in weight of the viscera during inanition were in general agreement with previous observations (Ott, 1924) Most of the intestines contained faeces of liquid consistency with a 'xanthophyll'/carotene ratio of 4/1 as compared with the normal faecal value of 2 The vitamin A level of the liver had not fallen very much, but the eyes and the kidneys had been depleted, 97% of the total vitamin was found in the liver

The results recorded in Tables 16 and 17 need little discussion Perhaps the major point is that despite the great changes incidental to prolonged fasting the overall X/C quotient for the whole frog rises to 4.6 exactly as in normal animals (3.6-4.6) over the same period of the year This seems to eliminate dietary influence and supports the idea of selective utilization of different carotenoids

Table 16 *Fat, carotenoids and vitamin A in female frogs kept in captivity for 25 weeks*

(Average decrease in body weight 35% Means not measured)

Organ	Average wt of organ as percentage of body wt *	Fat (mg /frog)	Vitamin A (μ g /frog)	Carotenoid (μ g /frog)	Xanthophyll Carotene
Liver	0.99	1.29	24.7	7.4	1.97
Eye	1.34	0.72	0.62	—	—
Kidney	0.23	1.21	0.074	0.5	2.09
Skin	9.01	3.39	—	19.2	2.71
Ovaries	5.26	17.0	—	22.1	10.2
Immature ovaries	1.19	2.17	—	8.7	19.0
Oviducts	8.30	5.19	—	1.3	0.78
Immature oviducts	0.94	—	—	0.3	3.37
Tongue	0.83	—	—	0.15	4.92
Pancreas	0.025	—	—	0.15	11.0
Lungs	0.274	—	—	0.13	—
Leg muscle	9.47	3.3	—	4.8	7.25
Oesophagus	0.31	—	—	0.12	—
Stomach	0.75	—	—	0.4	1.78
Intestine	0.43	—	—	0.2	6.37
Large intestine	0.19	—	—	0.05	—

* Compare Table 9 column 2

Table 17 *Distribution of 'xanthophyll' among the free form, mono and di esters in the organs of fasted female frogs*

Organ	Percentage of total 'xanthophyll' existing as		
	Di-ester	Mono-ester	Free form
Liver	8	18	74
Leg muscle	4	11	85
Skin	69	19	12
Oviducts	25	15	60
Ovaries	37	47	16
Immature ovaries	26	60	14

GENERAL COMMENTS

The present study perhaps makes timely a general comment on the place of distribution studies in the growth of ideas on the biological significance of particular compounds. It is a very uncertain place, the pattern of distribution is a resultant of superimposed patterns, of intake, assimilation, storage and utilization, of precursors and metabolites, and of enzyme capacities and 'detoxication' processes. Sometimes distribution studies seem merely to add new difficulties, but at other times they suggest fruitful lines of attack. A few examples may be given.

Vitamin A The distribution data are consistent with storage in the liver and with localized function in the eyes, but the apparent absence of vitamin A from normal nerve tissue, bone and mucosa is surprising in the light of the pathology of avitaminosis A. Comparative biochemistry adds to the complexity, e.g. the basking shark is found to store no vitamin A in its liver and the tunny fish to store enormous amounts.

Cholesterol The concentration of cholesterol in the adrenals pointed to functions, the nature of which is clarified by the effects of stress or injections of adrenocorticotrophic hormone. On the other hand, the broad picture of cholesterol distribution merely emphasizes the inadequacy of existing knowledge.

Provitamin D The occurrence of 7 dehydro cholesterol in skin fat is now seen to be significant, but its presence in ripe frog ovaries, in intestinal mucosa and in the sterols from worms is no easier to understand than the high concentration of ergosterol in yeast.

As was implied at the outset, this investigation was prompted by the idea that carotenoids may have functional significance in at least some animals. The advantage of the frog is that carotenoids are present at all stages in the life cycle, its disadvantage is that possible clues may be masked by the casual ecological variables and by shifting enzyme capacities. In the present state of carotenoid biochemistry the need is to find a bodily site or a developmental phase marked out by the distribution as possessing special interest.

Research in the field is halted by the lack of serviceable hypotheses. The present work suggests that (a) beneath much that may be casual there are strong suggestions of significance, (b) in both males and females, the sexual cycle exhibits patterns of storage, transfer and disappearance of carotene and xanthophyll, consistent with independent and significant roles, (c) the whole method of approach is in essence exploratory and its aim is to provoke the experiments which turn surmises into certainties.

SUMMARY

1 The occurrence of carotenoids and vitamin A in frogs (*Rana temporaria*) and tadpoles has been studied and new metrical data have been obtained on size and weight

2 Analytical methods for the accurate and speedy determination of carotenoids and vitamin A have been described and tested

3 Whole tadpoles were studied at different stages of development and the changes in total 'xanthophyll' and carotene content recorded. The presence of chlorophyll is ascribed to green food undergoing digestion. The 'xanthophyll'/carotene quotient declines from about 9 to about 3 as development proceeds. Vitamin A was detectable in tadpole eyes

4 The weights of frog organs expressed as per centage of body weight are recorded and maximum and minimum values given when variations are considerable. Much of the carotenoid actually ingested is contained in undigested food present in the alimentary tracts of insects or other prey. The frog does not ingest preformed vitamin A but converts carotene to the vitamin. About 85 % of the total vitamin occurs in the liver (mainly as ester), the remainder is normally about equally divided between kidneys and eyes. Frog kidneys contain a higher proportion of vitamin A than do mammalian kidneys. Most of the

vitamin A in the eye is ester, stored in the choroid. Carotenoids are dispersed over most organs but quite unevenly. The deposition of carotenoids in the ovaries is so large that a marked difference results between the sexes in carotenoid storage and utilization.

5 Mature ovaries contain in relatively high concentration a substance showing the four absorption maxima characteristic of 7 dehydrocholesterol and ergosterol.

6 Data have been obtained on the seasonal variation in different organs of some or all of the following: carotene, 'xanthophyll' (free, mono-ester, di-ester), vitamin A (free and esterified) and total fat. The results on normal and fasting frogs reveal (a) the retention of liver reserves in hibernation and in anation, (b) well defined seasonal variations in the carotenoid storage of different organs, (c) some indication of the continuance of carotenoid metabolism during hibernation, (d) the overwhelming quantitative importance of the ovarian requirements, (e) a drain on vitamin A reserves during reproduction, (f) the distinct patterns of storage and utilization of vitamin A, carotenes, 'xanthophylls' and fat in the frog.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance. One of us (D G R) held an Evans, Lescher and Webb Studentship and later a Johnston Fellowship in the University of Liverpool.

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A Histological Method for the Biological Estimation of Vitamin A

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(Received 19 April 1949)

There are considerable differences between physico-chemical and biological results of vitamin A estimations, owing to the fact that the 'analyst determines the proportion of a substance present, whilst the bio-assayist determines an effect' (Morton, 1942). The biological vitamin A value of food or pharmaceutical products is the sum of the values of all substances present having vitamin A activity, such as preformed vitamin A, either as the free alcohol or esters, and various carotenoid pigments. The response of the experimental animal to a given dose of test substance is dependent upon the absorption and utilization of vitamin A and its precursors, and is influenced by the digestibility of the food and the absence or presence of antioxidants. Physicochemical estimations of the vitamin A content of a product, on the other hand, depend in the first instance on determinations of intensity of absorption in the region 325–328 m μ where vitamin A shows its maximal light absorption. Allowance may have to be made for irrelevant absorption.

To convert extinction coefficients, as determined by physico-chemical means, to biological units, a conversion factor has been adopted. This factor, however, is chiefly based on the results of the curative 'growth' method (Coward, 1947) which, although not specific for vitamin A, is more accurate than methods based on examination of the vagina or the eye (xerophthalmia). The results of various doses of vitamin A upon pathological changes in the central nervous system of the rat led Irving & Richards (1940) to suggest that such changes might form the basis of a prophylactic assay of vitamin A. At weaning, rats were placed on a vitamin A-deficient diet and graded doses of the vitamin were administered. When after 7 weeks the rats were killed, degeneration was found in those receiving 1 i.u. or less daily, but not in those receiving 1.5 i.u. or more, and the demarcation was extremely sharp.

The work presented here was undertaken primarily to determine the suitability of the method suggested by Irving & Richards as a prophylactic assay. Arising from this, the accuracy of the present day values of the conversion factor was also investigated.

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Degeneration in the central nervous system due to vitamin A deficiency

Degeneration of the central nervous system in young swine, as a specific symptom of vitamin A deficiency, was described as early as 1914 by Hart & McCollum (1914, cf. Hart, Miller & McCollum, 1916). Their ration contained a high proportion of cereals, and it was believed that cereal toxicity was the cause of nervous degeneration. An unknown factor (vitamin A) 'associated with butter fat and certain other fats and found in considerably higher concentration in the leaves of alfalfa' had a protective action against nervous lesions.

Mellanby (1926) reported severe degeneration of the nervous system in puppies placed on high cereal diets, and stressed the toxicity of cereals in the absence of fat soluble vitamins. He suggested the name 'toxamin' for the harmful substance and ascribed its toxicity to interference with calcification of bones. His belief in the toxicity of cereals as the cause of degeneration was held for many years (Mellanby, 1930, 1931, 1934). Subsequent workers have not, however, supported it. Hughes, Lienhardt & Aubel (1929), who described nerve degeneration in pigs resulting from avitaminosis A, stated 'In no case, so far as we are aware, has anyone reported the finding of pathological changes in the nervous system accompanying avitaminosis A'. Zimmerman (1933) produced lesions in the nervous system with a diet containing no cereals. Finally, Irving & Richards (1938) replaced the cereal component of the diet by a mixture of tapioca starch and pure glucose (2:1) and proved that the belief in the toxicity of cereals was not justified.

Age is an essential factor in the production of nerve lesions in vitamin A deficiency. The main factors are the amount of vitamin A stored in the animal body at the beginning of the experiment and the effect of rapid growth in the early stages. This has been found by Suzman, Muller & Ungley (1932), Eveleth & Biester (1937), Wolbach & Howe (1925) and Wolbach & Bessey (1940, 1941).

That loss of weight alone does not account for paralysis or degeneration in the cord, was shown by several workers (Aberle, 1934; Wolbach & Bessey, 1940, 1942). Rats received a diet adequate in

vitamin A but restricted in amount, so that their weights paralleled those of litter mates on a vitamin A deficient diet. All the rats died after approximately the same experimental period, but, in contrast with the controls, those receiving vitamin A or carotene showed no paralysis or degeneration of the cord. It was also demonstrated that neither toco-pherol (Wolbach & Bessey, 1942) nor unsaturated fatty acids (Zimmerman, 1933, Zimmerman & Cowgill, 1936) affects the development of nervous lesions.

The general picture of vitamin A deficiency degeneration in the central nervous system of the rat has been described by Irving & Richards (1937, 1938). Maximum degeneration was usually found in the funiculus praedorsalis at the level of, or just below, the pyramidal decussation. The degeneration gradually became less, and more diffusely spread, at lower levels in the cervical cord. Microscopical examination of a cross section revealed the site of maximal degeneration in the anterior columns. As the severity of the lesions increased, the posterior columns and especially the column of Burdach, and the spino cerebellar tracts, gradually became more affected, though the degeneration never reached the same intensity as in the funiculus praedorsalis. Irving & Richards (1937) stated that the 'uniformity with which the condition described occurs in every one of the rats examined is in striking contrast with the variability in pathological condition, and in the occurrence of epithelial metaplasia found in animals in advanced avitaminosis A. The fact that this degeneration in the medulla occurs with such uniformity before the weight curve is affected to any extent, suggests that it is one of the fundamental lesions of vitamin A deficiency.'

The causation of nerve degeneration in vitamin A deficiency. No worker has so far produced a theory which satisfactorily accounts for this. That vitamin A plays an important role in calcification of bones was pointed out by Copp & Greenberg (1945). They found that fracture callus was much smaller in the deficient rat than in the normal animal and the calcification was less active. Mellanby (1938, 1941, 1947) postulated that vitamin A deficiency upset the regulation of skeletal growth by means of interference with the activity of the osteoblasts and osteoclasts. On a surface where osteoclasts were normally active, their activity could be suppressed and that of the osteoblasts might predominate and vice versa. The resulting bony overgrowth caused pressure on the central nervous system and peripheral nerves by squeezing and stretching and resulted in degeneration due to mechanical injuries.

Wolbach & Bessey (1940, 1941, 1942) and Moore & Sykes (1941) agreed with Mellanby that degeneration was caused by mechanical injuries due to pressure. They, however, ascribed this to causes other

than bone growth. The bones ceased to grow with the establishment of vitamin A deficiency, which caused a disproportionate growth of the central nervous system in relation to its bony enclosure. Attention was also drawn to the possibility that vitamin A deficiency accelerated the growth of the central nervous system (Wolbach & Bessey, 1940).

Another factor that needs to be considered here is the increase in spinal fluid pressure that accompanies vitamin A deficiency, as was found by Moore & Sykes (1940, 1941) in bovines. It was suggested by these workers that incoordination and nervous lesions might be related to increases in the cerebrospinal fluid pressure. This was due to an increased volume of the cranial contents, caused by either a relative overgrowth as indicated by Wolbach & Bessey, or to bone overgrowth as postulated by Mellanby.

These data indicate that, whatever the mechanism of vitamin A deficiency degeneration may be, it is still obscure. As far as the hypothesis of cessation of bone growth is concerned, the work of Orr & Richards (1934) and of Irving (1948) disproves it. Bone overgrowth and increased intracranial pressure have been definitely demonstrated. If, however, degeneration was due to any kind of pressure effect, an even distribution of demyelinated fibres would be expected in a cross section of the cord and not the well defined degeneration that has been found always to occur in the same tracts.

EXPERIMENTAL

Diet. The composition of the vitamin A free diet was as follows: Casein, 17, potato starch, 47, arachis oil, 14.1, brewer's yeast, 17.2, salt mixture (McCollum 1935), 4.7%.

Owing to post-war restrictions it was impossible to obtain cornstarch and so potato starch was used instead. The arachis oil was obtained in one gallon tins and was of a good quality. The colour varied from a light straw colour to light brown and no vitamin A or carotenoids could be detected in it by the SbCl_3 test. Before using a new tin it was fortified with calciferol so as to obtain approximately 100 i.u. vitamin D/kg of mixed diet. It contained enough toco-pherol to give maximum vitamin A protection. The casein was extracted with 96% ethanol for about 7-8 hr before using, care being taken not to overheat the casein during this treatment, in order to avoid diarrhoea in the experimental animals. It is said that rats require no ascorbic acid, but to be on the safe side one ground tablet, equal to 50 mg vitamin C, was included in every 2 kg of diet. The vitamin A free food mixture (without the arachis oil) was mixed in quantities of 3 kg at a time. Each day the necessary amount of this dry food mixture was weighed out and the corresponding amount of KI solution (0.64 g/l) added, namely 0.5 ml for every 70 g of the former. The oil was rubbed in and afterwards enough water was added to make a stiff paste, which was crumbled up and put into the food pots.

Rats. Young rats of the Wistar Institute strain were employed. Litters of more than nine were reduced to that number at birth. The whole litter, including the mother, was

placed on the vitamin A free diet 5 days before weaning, which was done at 22 days of age. This procedure had been adopted by Irving & Richards (1938), who found that it produced more consistent results, and that the test animals so treated showed signs of deficiency 7 or more days before those not put on the deficient diet before weaning.

As a general rule, the litters contained more females than males. To save time and material, two different preparations were tested simultaneously against the same standard and with three groups for every oil solution, each assay consisted of nine dosage groups. With a limited number of litters, it was not always possible to start such an experiment with equal numbers of males and females (5, 5) in each group. The distribution was, therefore, always carried out with the main object of starting an experiment with ten rats in each group which consisted as far as possible of five males and five females and to minimize the influence of litter variations.

Oils tested. Biological experiments were carried out on samples of the following, the spectroscopic characteristics (Table 8) of which were also determined first:

- (1) Vaalhaai (*Galeorhinus capensis*) liver oil
- (2) Vitamin A acetate in arachis oil
- (3) Vitamin A distillate in arachis oil
- (4) Snoek (*Thyrates atun*) liver oil
- (5) Hake (*Merluccius capensis*) liver oil.
- (6) Tuna (*Germo albacora*) liver oil
- (7) Dogfish (*Squalus* species) liver oil
- (8) Stonebass (*Polyprion americanus*) liver oil

Except for the vitamin A acetate and distillate the rest were natural fish liver oils, where the vitamin A exists chiefly in the form of esters. Determinations of extinction coefficients were made with the Beckman spectrophotometer using cyclohexane as solvent and only in the case of the dogfish liver oil was the estimation carried out on the unsaponifiable fraction. The vitamin A acetate and the distillate were dissolved in arachis oil to an E value of about 7 and the spectrophotometric estimations were carried out on the solutions.

Conduct of the experiment. To obtain the final dosing solutions, the oils were diluted in steps with arachis oil to a potency of about 60 i.u./g. as calculated from the $E_{1\text{cm}}^{1\%}$ values using 1600 as conversion factor. Fresh dosing solutions were made up every 2 weeks from a solution of intermediate strength. All the oils were stored in the refrigerator in glass stoppered bottles. International β carotene standard and vitamin A acetate were used as biological standards and each experiment was a comparison between standard and test oil responses which were obtained simultaneously. The solution of crystalline vitamin A acetate was made up in pure arachis oil to an $E_{1\text{cm}}^{1\%}$ value of 6.85. This was used as a standard of reference in a further diluted form after standardization against the International β carotene standard. Thus the β carotene preparation was accepted as the primary standard throughout. The U.S.P. vitamin A acetate standard was not used. The acetate referred to was a crystalline preparation obtained commercially.

Dosing started at the beginning of the experiment and vitamin A was administered orally every 3 days by means of a tuberculin syringe with a micrometer screw attachment, which released a standard drop of 0.01 g. (= 0.6 i.u. vitamin A) per turn. Preliminary experiments showed that satisfactory results were obtained when vitamin A was administered every third day. The different dosage groups re-

ceived, therefore, 1, 2 and 4 turns per rat every third day, which was equal to approximately 0.2, 0.4 and 0.8 i.u. of this vitamin per day. These three levels were selected since it was expected that the dosage response curve would be a straight line when the response was plotted against the log of the dose. Owing to the possibility that the time of dosing might have an influence on the absorption and utilization of vitamin A and β carotene, dosing was always carried out before feeding. The test period lasted for 35–42 days and the weights and eye conditions were taken as criteria as to when to stop the experiment, when the rats were killed with coal gas.

Histological examination of the central nervous system. In the demonstration of degeneration in the nervous system, most workers prefer to use the Marchi method, whilst Kultschitzky's stain and polarized light (Lee & Surc, 1937, Setterfield & Sutton, 1935) are less often applied. In the present experiments the Marchi technique was employed. Irving & Richards (1938) showed that, if proper precautions were taken, this method gave reliable and reproducible results, the method employed followed in the main that adopted by them. Though the Marchi technique is usually considered rather slow, a procedure was evolved whereby it was possible to embed eighty medullas in 1 day. A résumé of the method is therefore given for the benefit of other workers who may use this method of assay.

Rats were killed with coal gas in groups of about ten each. Part of the skin and the muscles on the back were removed to expose the skull and upper cervical vertebrae but with out removing the ears, which had distinctive markings. The cranium and spinal canal were opened with great care to avoid injuring the medulla. Having finished that operation, the skull with the central nervous system inside was cut off from the rest of the body. Owing to the tension of the ligaments the cervical vertebrae were then pulled at an angle to the skull. To avoid fixing the medulla in that right-angled position, the axis of the skull was aligned with that of the spinal cord by inserting a sharpened match stick through the trachea into the mouth.

The skulls of the rats belonging to different dosage groups were placed in bottles containing 2.5% (w/v) aqueous $K_2Cr_2O_7$ solutions with corresponding labels. After 5 days the dissection was completed by removing the lower medulla and upper cervical cord (subsequently referred to as the medulla), which was transferred to the same strength of $K_2Cr_2O_7$ solution in a cork stoppered, flat-bottomed specimen tube, where it remained for a further 5 days. The nervous tissue was very soft at that stage, and it was necessary to handle it with the utmost care to avoid squashing it between the fingers.

The 2.5% $K_2Cr_2O_7$ solution was next poured out and about 3 ml. of a mixture consisting of one part of a 1% (w/v) osmic acid solution in two parts of a 5.3% (w/v) solution of $K_2Cr_2O_7$, thoroughly mixed in a glass stoppered measuring cylinder, was put into the tube containing the specimen. That solution was changed for a similar one after 5 days for the same period. The tissues were then washed, dehydrated in ethanol, cleared in $CHCl_3$ and embedded in paraffin.

Sections (10 μ) were cut serially starting from the cord end. Two out of every ten were taken and arranged in sequence on the microscope slide in two parallel rows of ten each. As a general rule, Marchi stained nervous tissue is very brittle, but the use of the following precautions seemed to improve the method, and sections without any cracks

were obtained. Care was taken to perform the transference of the medullas from the CHCl_3 to the wax baths as quickly as possible, lest the tissues should dry and cracks appear by shrinkage. A vacuum oven was used for the embedding and it was found important to decrease the pressure very gradually as a rapid decrease also resulted in cracks.

Criterion of response. Irving & Richards (1938, 1940) indicated the degree of severity of the nervous lesions by numbers according to the following scheme: stage 1—, a very few fibres degenerated, stage 1, a few scattered fibres degenerated, stage 2, well marked degeneration, stage 3, very marked degeneration, stage 4, heavy degeneration.

They added that the degree of degeneration represented by the numbers of 1 and 1— was of no significance, since an exactly similar effect was observed in positive controls and stock rats, and was an artefact of the Marchi technique. This experience was not confirmed in the present work. No degenerated fibres could be detected under the low power of the microscope in stock rats and positive controls, and the slightest degeneration found in the experimental rats was, therefore, taken as an indication of the first vitamin A-deficiency symptom.

The classification used by Irving & Richards was found to be unsuitable for assay purposes since statistical methods could not be applied. It was found, however, that with a little experience the degeneration could be divided into six different stages, thereby increasing the accuracy of the estimation.

With the object of obtaining a positive curve of response it was decided to use the degree of protection against degeneration as the criterion of response. The value 0 was assigned to a section showing maximal degeneration to indicate that the degree of protection against degeneration was 0 in this case. A section with no degenerated fibres received the value 5 indicating full protection. (See Figs 3–8, Pls 6 and 7.)

For the classification of the slides, two microscopes with mechanical stages were used. Slides belonging to the same assay were first classified roughly with one microscope in different groups according to their 'stage of protection'. A standard slide, say of stage 4, was next put under one microscope and slides which were expected to show approximately the same protection were examined individually under the other microscope. By finding first the section in

Table 1 *Estimated degree of protection against myelin degeneration in male rats given by different doses of vitamin A*

(Each numbered experiment gives the result of assays carried out simultaneously. The International Standard was the β carotene standard preparation, as issued by the National Institute for Medical Research, which was used after further dilution with pure arachis oil to a potency of 60 i.u./g. The vitamin A acetate solution ($E_{1\text{cm}}^{1\%}$ 685, see p. 630) was prepared from crystalline material. The letters (a), (b) and (c) below and in the following tables refer to duplicate or triplicate assays carried out on the same preparation. The potency of liver oils was roughly assessed by the gross $E_{1\text{cm}}^{1\%}$ value $\times 1600$ D.P. = degree of protection.)

Exp no	Preparation	Dose					
		0.2 i.u./day		0.4 i.u./day		0.8 i.u./day	
		No males	D.P.	No males	D.P.	No males	D.P.
1	Internat. Std. (a)	3	1.5	4	5.0	4	10.0
	Vaalhaai liver oil (a)	3	2.0	3	4.5	4	9.5
2	Internat. Std. (b)	2	1.5	4	2.0	4	4.5
	Vaalhaai liver oil (b)	2	0.5	4	2.5	4	6.5
3	Vit. A acetate (a)	3	6.5	4	11.5	4	14.0
	Internat. Std. (a)	3	7.0	4	9.5	4	16.0
	Vit. A distillate (a)	3	8.5	4	9.0	4	16.5
4	Vit. A acetate (b)	5	10.0	5	10.5	5	16.5
	Internat. Std. (b)	5	9.5	5	9.5	5	15.0
	Vit. A distillate (b)	5	12.5	5	14.0	4	15.5
5	Snoek liver oil (a)	4	3.5	4	7.5	5	13.0
	Vit. A acetate Std. (a)	4	1.5	5	9.5	4	10.0
	Hake liver oil (a)	3	1.5	4	7.5	5	16.5
6	Snoek liver oil (b)	6	4.0	6	10.0	5	10.5
	Vit. A acetate Std. (b)	6	5.5	5	6.0	5	18.5
	Hake liver oil (b)	6	7.0	6	10.0	5	15.0
7	Tuna liver oil (a)	5	6.0	5	12.0	5	19.0
	Internat. Std. (a)	5	6.0	5	11.5	5	19.0
	Dogfish liver oil (a)	4	4.5	5	8.5	5	19.0
8	Tuna liver oil (b)	5	2.5	5	10.5	5	14.5
	Internat. Std. (b)	5	7.0	5	8.5	5	15.5
	Dogfish liver oil (b)	5	4.0	4	4.5	5	11.0
9	Stonebaas liver oil (a)	5	1.5	4	2.0	5	13.0
	Vit. A acetate Std.	6	4.5	6	5.5	6	18.5
	Stonebaas liver oil (b)	4	2.5	5	8.5	4	8.0
	Vaalhaai liver oil (c)	4	2.0	4	5.0	5	12.5
Totals		111	123.0	120	205.0	121	357.5
Weighted means			1.108		1.708		2.954

the two rows revealing the maximal degeneration and, secondly, by comparing the number and size of the black areas in this section with those of the standard under the first microscope it was easy to recognize the actual stage of protection. If the black spots showed a similar intensity to that of the standard, that specific slide was taken as stage 4, while a little more degeneration denoted stage 3.5 and a little less stage 4.5. This procedure was repeated for every group. After a little experience had been gained, it was found that the classification of the same slides undertaken a few weeks later showed no significant difference.

RESULTS

Difference in response of males and females
Coward (1932) described the difference in response to vitamin A dosage of male and female rats. She found that males gave results with a greater standard deviation than that given by female rats. However, the steeper slope of the curve of response of the former caused them to give results of greater accuracy than those obtained with female rats. Brenner, Brookes & Roberts (1942) observed a significant sex difference in the liver storage of vitamin A in rats. 'The females stored and retained more vitamin A in the liver than the males, while the

males had higher blood values'. Using the fluorescence technique to demonstrate vitamin A in the

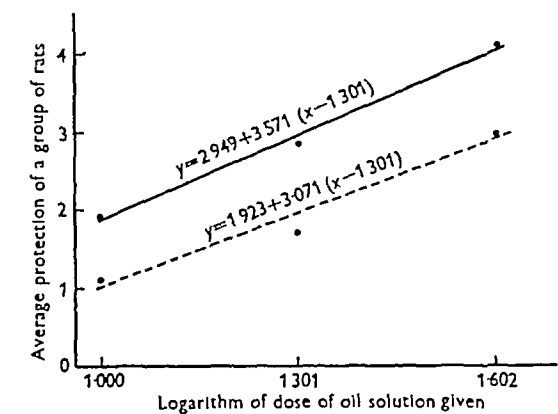


Fig. 1. Curve relating the degrees of protection of the central nervous system in male and female rats to the logarithm of the dose of vitamin A. —, females, ---, males.

liver, Popper & Brenner (1942) came to the conclusion that 'During depletion the livers of male rats

Table 2. *Estimated degree of protection against myelin degeneration in female rats given by different doses of vitamin A*

(See sub heading to Table 1)

Exp no	Preparation	Dose					
		0.2 i u/day		0.4 i u/day		0.8 i u/day	
		No females	D.P.	No females	D.P.	No females	D.P.
1	Internat. Std. (a)	6	6.0	5	17.0	5	21.0
	Vaalhaai liver oil (a)	6	12.0	5	10.5	5	20.5
2	Internat. Std. (b)	5	4.0	4	8.5	4	15.5
	Vaalhaai liver oil (b)	5	5.5	4	4.5	4	8.5
3	Vit. A acetate (a)	8	21.0	6	21.5	6	24.5
	Internat. Std. (a)	8	25.5	6	20.5	6	23.5
	Vit. A distillate (a)	8	23.5	6	18.0	6	25.5
4	Vit. A acetate (b)	5	11.0	5	17.5	5	22.0
	Internat. Std. (b)	5	14.5	5	19.5	5	22.0
	Vit. A distillate (b)	5	10.5	5	18.0	5	20.0
5	Snoek liver oil (a)	6	11.0	6	15.5	5	12.0
	Vit. A acetate Std. (a)	6	8.0	5	12.5	5	21.0
	Hake liver oil (a)	6	10.5	6	19.5	5	20.5
6	Snoek liver oil (b)	4	3.5	4	9.5	5	20.0
	Vit. A acetate Std. (b)	4	0.0	5	5.5	5	22.0
	Hake liver oil (b)	4	9.0	4	11.0	5	23.5
7	Tuna liver oil (a)	5	12.0	5	13.5	5	22.5
	Internat. Std. (a)	5	14.0	5	17.5	5	21.5
	Dogfish liver oil (a)	5	8.5	5	15.5	4	17.5
8	Tuna liver oil (b)	5	7.5	5	13.5	5	22.0
	Internat. Std. (b)	5	7.5	5	15.5	5	18.5
	Dogfish liver oil (b)	5	8.0	5	11.5	5	18.5
9	Stonebass liver oil (a)	5	2.0	6	17.5	5	20.5
	Vit. A acetate Std.	6	12.0	5	13.5	6	25.5
	Stonebass liver oil (b)	6	8.5	5	10.0	6	25.5
	Vaalhaai liver oil (c)	6	9.0	6	13.0	4	13.0
Totals		144	276.5	133	380.0	131	533.0
Weighted means			1.920		2.857		4.089

lose vitamin A faster than those of females. There is, however, no sex difference with respect to method of utilization, as judged from the histologic picture. These observations are in accord with the results obtained with male and female rats as demonstrated in Tables 1 and 2.

The dosing solutions were made up so as to contain approximately 0.6 i.u. of vitamin A per 10 mg. When dosing every 3 days, the 0.2 i.u. per day group of rats received 10 mg. of this oily solution, while the 0.4 and 0.8 i.u. per day groups were given 20 and 40 mg., respectively. The degrees of protection of the individual rats were added together and appear in columns 4, 6 and 8.

By plotting the average results obtained in Tables 1 and 2 against the logarithms (to the base 10) of the doses the curves of response proved to be straight lines (see Fig. 1).

The average protection of females against degeneration was, however, $2.949/1.923 = 1.533$ times that of the males and it was, therefore, justifiable to assume that the minimum vitamin A requirement of male rats (about 9 weeks of age) was about 1.5 times that of females of the same age. Several workers have shown that the vitamin A requirement is a function of the body weight (Guilbert & Hart,

1935, Hart, 1940, Callison & Knowles, 1945, Paul & Paul, 1946). However, the latter alone cannot account for the sex difference in the daily requirement of this vitamin, or vice versa for the sex difference of protection against degeneration, since it is evident from the figures in Table 3 that the ratio of the body weights of male and female rats bears no relation to the protection ratio.

Curve of response. The sex difference in the response of rats to the same dose of vitamin A created a difficulty. It was impossible to use one sex only owing to the limitations in the breeding facilities. Two alternatives to solve the problem remained, namely, to keep the females longer on experiment until they reached a degeneration stage equal to that of the males, or to use equal numbers of males and females in all the groups. The former possibility was considered impracticable owing to the fact that it took more than 3 weeks to determine the stage of degeneration. With limited numbers of rats the latter alternative could not be followed.

To overcome this trouble of sex difference and to obtain results which might lend themselves readily to statistical analysis, Prof. S. J. Pretorius of Stellenbosch University suggested personally that an average factor should be calculated for each experi-

Table 3. Average weights of rats at the end of the test period

Preparation	Dose					
	0.2 i.u./day		0.4 i.u./day		0.8 i.u./day	
	Males (g.)	Females (g.)	Males (g.)	Females (g.)	Males (g.)	Females (g.)
Internat. Std. (a)	99	85	112	89	108	90
Vaalhaai liver oil (a)	101	84	111	87	102	93
Internat. Std. (b)	86	83	92	87	109	89
Vaalhaai liver oil (b)	84	85	97	84	113	87
Vit. A acetate (a)	135	105	140	99	151	116
Internat. Std. (a)	135	108	145	104	143	110
Vit. A distillate (a)	115	107	142	108	155	109
Vit. A acetate (b)	74	74	79	73	91	74
Internat. Std. (b)	70	73	80	70	94	82
Vit. A distillate (b)	72	76	78	77	98	74
Snoek liver oil (a)	81	86	100	83	113	85
Vit. A acetate Std. (a)	75	85	102	79	91	103
Hake liver oil (a)	85	82	95	86	103	88
Snoek liver oil (b)	88	69	94	83	109	90
Vit. A acetate Std. (b)	91	81	100	90	112	94
Hake liver oil (b)	93	79	91	84	105	87
Tuna liver oil (a)	100	80	102	85	111	88
Internat. Std. (a)	96	84	100	92	112	85
Dogfish liver oil (a)	99	81	109	89	112	88
Tuna liver oil (b)	91	81	99	84	106	94
Internat. Std. (b)	100	86	96	95	107	95
Dogfish liver oil (b)	93	81	105	89	111	99
Stonebass liver oil (a)	75	84	91	89	106	90
Vit. A acetate Std.	90	81	95	85	113	96
Stonebass liver oil (b)	88	82	84	89	114	81
Vaalhaai liver oil (c)	85	80	86	91	121	93
Totals	2441	2179	2626	2271	2910	2380
Averages	94	84	101	87	112	91
Ratio males/females		1.12		1.16		1.22

ment by which to multiply the response of every individual female in that assay, so as to obtain a response corresponding to that of a male or vice

Table 4 *Estimated protection against myelin degeneration given by different doses of International Standard β carotene*

(Protection is expressed in terms of the results on single rats, transformed as indicated in text to eliminate sex differences)

	Daily doses of International Standard		
	0.2 i u D.P	0.4 i u D.P	0.8 i u D.P
	1.0	1.5	3.5
	1.0	3.0	3.0
	0.5	1.5	3.5
	2.0	3.0	4.5
	1.5	2.5	4.5
	2.71	3.05	2.71
	0.68	3.05	2.37
	1.70	1.70	3.39
	3.50	1.70	3.05
	1.36	2.37	3.05
	1.0	0.0	3.0
	2.0	2.0	3.5
	0.5	2.5	3.5
	1.5	2.0	2.5
	2.0	2.0	3.0
	1.26	1.90	2.84
	0.32	1.90	1.20
	1.26	0.95	2.52
	0.95	2.84	2.84
	0.95	2.21	2.21
Totals	27.24	41.67	60.74
Weighted means	1.362	2.0835	3.037

versa. Such a factor represents the ratio of average response of males to average response of females in the present experiments. This suggestion was tested out and gave satisfactory results. Such transformed

results were used in calculating the vitamin A potencies of the different oils.

The log dose response curve with this method was a straight line both for the β carotene standard (Table 4) and vitamin A containing oils (Table 5). The data of these two tables were used to plot the graphs in Fig. 2.

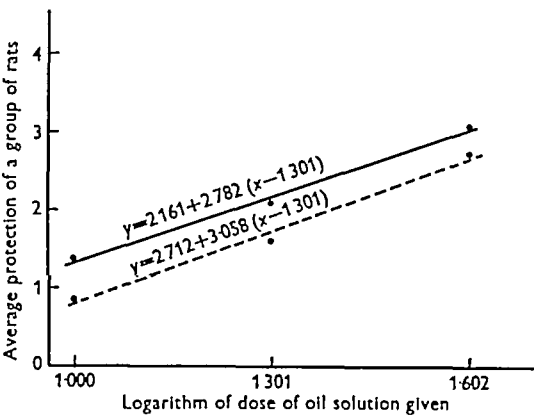


Fig. 2. Curve relating the degrees of protection of the central nervous system to the logarithm of the doses of β carotene standard and fish liver oils. —, β carotene standard, ---, fish liver oils.

Owing to the fact that the response of rats to a given dose of vitamin A is subject to fluctuations, it is absolutely essential that each test should be a comparison between the response given to a dose of the test substance and the response given to a dose of the standard at the same time (Coward, 1947, p. 2). Experimental rats must be distributed in equal numbers between the biological standard and the substances to be tested and the slope of the response curve must be obtained from every individual test.

Table 5 *Estimated degree of protection against myelin degeneration given by different doses of vitamin A*

Oil sample	Dose					
	0.2 i u		0.4 i u		0.8 i u	
	No. of rats	Value for group	No. of rats	Value for group	No. of rats	Value for group
Snoek (a)	10	9.76	10	16.34	10	24.96
Vit. A acetate Std. (a)	10	6.05	10	16.61	9	21.95
Hake (a)	9	7.47	10	18.63	10	28.18
Snoek (b)	10	6.17	10	15.89	10	22.93
Vit. A acetate Std. (b)	10	9.22	10	15.62	10	32.17
Hake (b)	10	12.58	10	16.82	10	29.62
Tuna (a)	10	14.15	9	21.15	10	35.25
Dogfish (a)	9	10.27	10	19.01	9	30.86
Tuna (b)	10	7.23	10	19.03	10	28.37
Dogfish (b)	10	9.05	9	11.75	10	22.67
Stonebass (a)	10	2.58	10	11.39	10	24.02
Vit. A acetate Std.	12	10.96	11	11.39	10	24.02
Stonebass (b)	10	7.09	10	13.80	10	21.71
Vaalhaai (c)	10	6.86	10	12.00	9	19.49
Totals	140	119.44	139	220.88	139	374.39
Weighted means		0.8531		1.5891		2.6984

itself. The slope from a previously constructed dosage response curve cannot be used, because 'large changes of slope may occur with time, and these changes do not occur in a random fashion but exhibit a secular or quasiperiodic tendency. The use of a fixed slope therefore introduces an uncontrolled error for which it is impossible to allow. Even if

results are summarized in Table 7. A comparison of the limits of error in this table and those given by Gridgeman (1944) and Irwin (1937) for the 3 weeks' curative 'growth' method indicates that this histological method for the bio assay of vitamin A is as accurate as and possibly more accurate than the 3 weeks' curative 'growth' test.

Table 6 *Fluctuations in the slopes of the log dose response curves*

Section no	Test oil	Standard	Test period	Slope *
1	Vaalhaai (a)	- Internat Std	2 iii. 47-18 iv 47	0.786
2	Vaalhaai (b)	- Internat Std	24 iii. 47-6 v 47	0.475
3	Vit A acetate (a)	- Internat Std	27 iv 47-29 vi 47	0.563
	Vit A distillate (a)	- Internat Std	27 iv 47-29 vi 47	0.547
4	Vit A acetate (b)	- Internat Std	1 vi 47-14 vii 47	0.635
	Vit A distillate (b)	- Internat Std	1 vi 47-14 vii 47	0.521
5	Snoek (a)	- Vit A acetate Std	21 vi 47-11 viii 47	0.838
	Hake (a)	- Vit A acetate Std	21 vi 47-11 viii 47	0.956
6	Snoek (b)	- Vit A acetate Std	21 vii 47-7 ix 47	0.993
	Hake (b)	- Vit A acetate Std	21 vii 47-7 ix 47	1.000
7	Tuna (a)	- Internat Std	12 viii 47-25 ix 47	0.954
	Dogfish (a)	- Internat Std	12 viii 47-25 ix 47	1.017
8	Tuna (b)	- Internat Std	14 ix 47-23 x 47	0.914
	Dogfish (b)	- Internat Std	14 ix 47-23 x 47	0.726
9	Stonebass (a)	- Vit A acetate Std	31 x 47-12 xi 47	0.970
	Stonebass (b)	- Vit A acetate Std	31 x 47-12 xi 47	0.815
	Vaalhaai (c)	- Vit A acetate Std	31 x 47-12 xi 47	0.819

* The values of these slopes are not of the same magnitude as those previously used

regular records of slopes are kept, and their standard deviation over a considerable period of time calculated, this does not get over the difficulty. For the variations are not random, and so this standard deviation gives no clue to the error of the slope at any particular time' (Irwin, 1944). This author demonstrated further that it is faulty to take the position and slope of the response curve given by the doses of vitamin A standard as correct and to use that for calculating the potency of the test substance. The correct procedure is to find the slope of the best fitting straight line through all the observed points, 'that is to say, it assumes that apart from sampling error the slopes provided by the standard and the test substance are the same. The horizontal distance between the parallel straight lines then provides an estimate of the potency ratio'.

A similar method of calculation was used in this work and Table 6 illustrates the fluctuations which were found in the slopes of the response curves.

The slopes found for every section show very good agreement, but significant differences exist between slopes of different assays, e.g. section no. 3 and section no. 6. These data therefore confirm the statement of Coward quoted above.

As the log dose response curve is a straight line, the statistical method described in the British Standards Institution Specification no. 911 (1940) was used to analyse the experimental data and the

Table 7 *Limits of error and conversion factors estimated for different vitamin A preparations*

(The average number of rats used in an assay of one oil was 30)

Oil sample	Conversion factor	Average conversion factor	Limits of error (%)
Vaalhaai (a)	1805	—	84.2-118.9
(b)	1659	—	51.1-185.3
(c)	1391	1618	74.3-130.9
Vit A acetate (a)	1818	—	52.6-151.2
(b)	1774	1796	54.6-179.5
Vit A distillate (a)	1455	—	63.6-156.2
(b)	2459	1957	67.9-202.2
Tuna (a)	1776	—	75.6-132.1
(b)	1684	1730	78.4-126.8
Dogfish (a)	1557	—	75.1-130.4
(b)	1265	1411	69.4-135.8
Snoek (a)	1788	—	75.9-134.0
(b)	1234	1511	71.8-134.0
Hake (a)	1951	—	73.5-141.6
(b)	1683	1817	75.7-133.0
Stonebass (a)	1408	—	78.9-124.5
(b)	1538	1473	77.0-128.0

The given doses of both the vitamin A standard and the test oil were always in geometrical progression. This did not apply to the total vitamin A intake, owing to the fact that the rats received small but equal amounts of carotene derived from the

potato starch. Such an extra allowance, although small, would obviously tend to interfere with the log dose response relationship. Therefore, a more satisfactory vitamin A free diet would probably increase the accuracy of this method.

Correction for irrelevant absorption. Until recently it was believed that the full intensity of absorption at 328 m μ was due to the presence of vitamin A. This assumption was proved to be unjustified, owing to the presence of irrelevant absorbing substances, even in high potency oils and their unsaponifiable fractions.

The influence of the correction for irrelevant absorption, as suggested by Morton & Stubbs (1946, 1948) on the conversion factors obtained in this work, is illustrated in Table 8.

The curative method is also carried out on animals which may differ widely in pathological condition, and this main source of error is avoided by using the prophylactic method. In this bio assay no animals need to be discarded.

SUMMARY

1 Degeneration in the central nervous system is a characteristic symptom of vitamin A deficiency in rats. The Marchi technique is sufficiently accurate for following such degenerative changes in the myelin substance of the central nervous system, provided that it is properly used. With slight modifications in histological procedure it can be used for quantitative routine work.

Table 8 *Influence of correction for irrelevant absorption on the conversion factor*

Preparation	Average* potency (i u/g)	<i>E</i>		Conversion factor	
		Observed	Corrected	Observed	Corrected
Vaalhaai liver oil	15,000	9.20	8.68	1618	1727
Vit. A acetate	12,300	6.85	6.80	1796	1810
Vit. A distillate	16,500	8.44	8.15	1957	2028
Tuna liver oil	129,900	75.10	67.50	1730	1925
Dogfish liver oil	5,710	4.05	3.60	1411	1586
Snoek liver oil	26,700	17.64	16.56	1511	1610
Hake liver oil	13,900	7.68	7.25	1817	1924
Stonebass liver oil	78,600	53.37	46.75	1473	1682
			Averages	1665†	1786

* International β carotene preparation accepted as primary standard throughout.

† The average value for the liver oils only was 1593.

It is evident from the data in Table 8 that a conversion factor of 1600 is preferable for uncorrected *E* values and that the conversion factor of 2000 which is generally used in America, is definitely too high. A higher conversion factor should be used for corrected *E* values and the value of 1800 suggested by Morton & Stubbs (1948) is supported by these data.

DISCUSSION

The main objection to the use of a prophylactic method is that it takes longer to perform than the curative test. Time, labour and money, however, are saved at the feeding stage of this assay, seeing that the test period is not longer than 6 weeks, whereas the depletion period of the curative method takes about 5 weeks and the test period is at least 3 weeks in addition. Thus, the experimental periods for this prophylactic method and the curative test are 6, and at least 8 weeks, respectively.

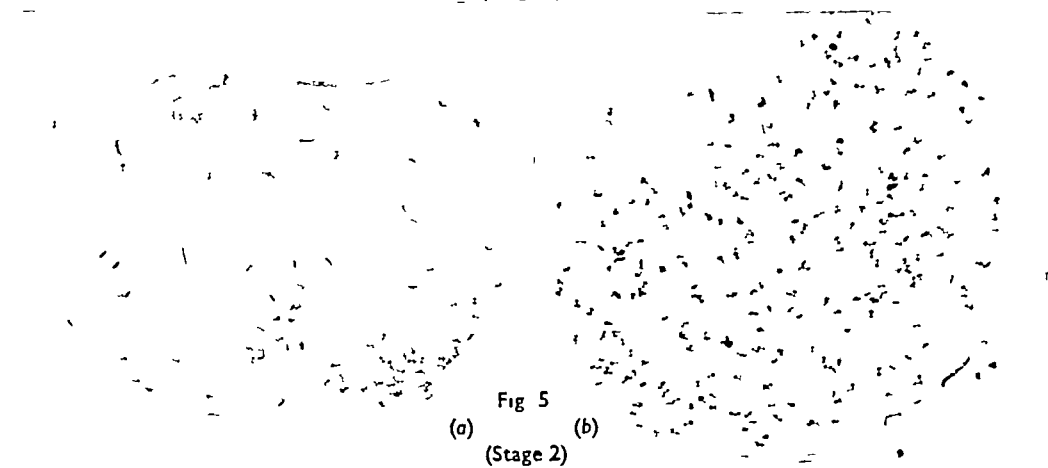
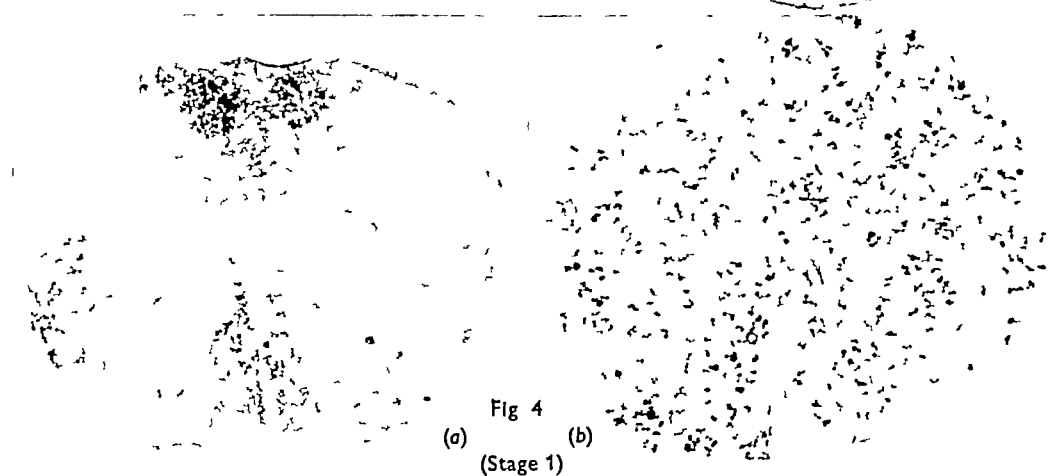
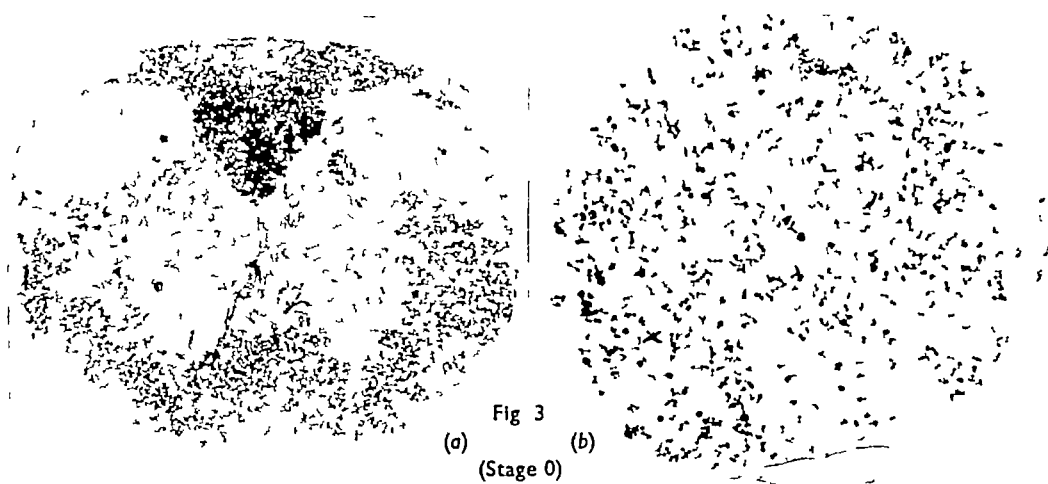
The curative method has the further disadvantage that frequently rats have to be rejected at the end of the preliminary period, because their weight curves render them 'unsuitable' for the test. A further reduction in the number of available animals may result owing to losses by death during the depletion

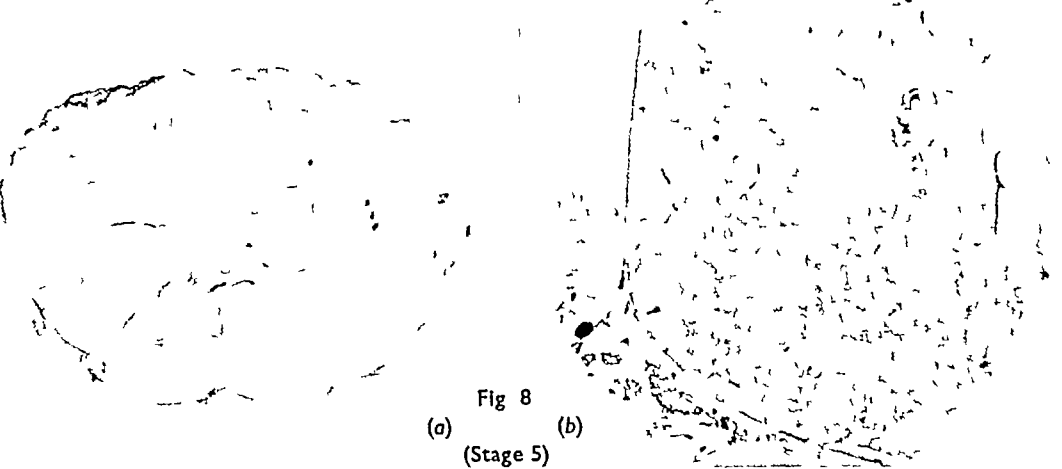
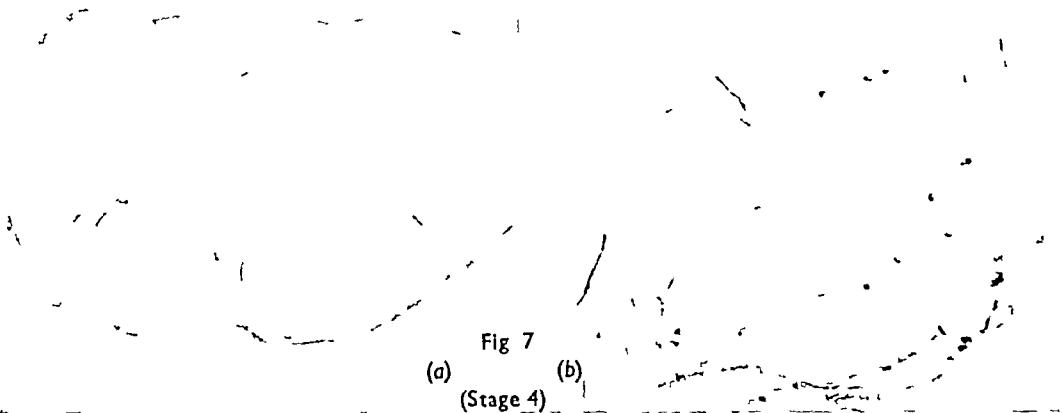
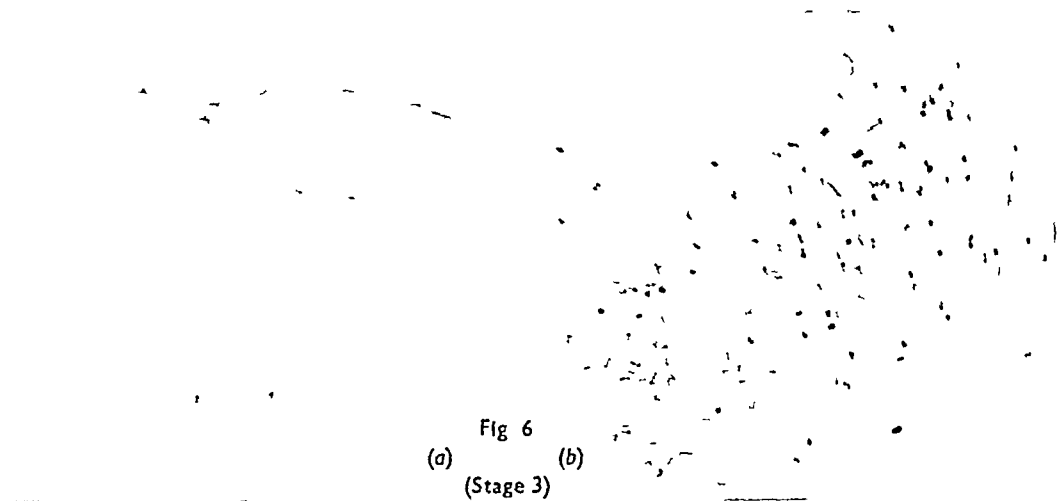
2 A histological method for the biological estimation of vitamin A is described. If groups of young rats are kept under the same experimental conditions and receive graded doses of vitamin A over a suitable range, equal proportional increases in doses produce approximately equal average increases in protection against degeneration in the central nervous system.

3 There exists a significant difference between the response (protection against degeneration) of male and female rats to the same dose of vitamin A. This difference indicates that the minimum vitamin A requirement of male rats is about 1.5 times that of females of the same age. Although it is generally assumed that vitamin A requirement is a function of body weight, the latter alone cannot account for the sex difference.

4 The vitamin A potencies of vitamin A acetate, vitamin A distillate and six natural fish liver oils were estimated by means of this histological method and corresponding conversion factors were calculated to test out the reliability of the present conversion factors.

5 The experimental data were analysed statistically with the result that the histological method seemed to be as accurate as, and possibly more accurate than, the 3 weeks' curative 'growth' test.





6 The calculated conversion factors indicate that 1600 is preferable to 2000 for uncorrected *E* values. A higher factor should be used for corrected *E* values.

The author wishes to express his gratitude to Prof J T Irving for his encouragement, guidance and continued interest in this work, to Dr H M Schwartz for her highly

esteemed advice and assistance, to Prof S J Pretorius for his advice in the carrying out of the statistical analysis, to Mr H Hall for taking the photomicrographs.

The author was awarded a research assistantship by the Council of Scientific and Industrial Research of South Africa to enable him to carry out this work, for this, grateful acknowledgement is made. The expenses of the research were also borne by the same body.

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EXPLANATION OF PLATES 6 AND 7

Gross sections of the medulla showing different stages of protection against degeneration.

Marchi stained sections of the cord do not lend themselves readily to photography, especially at lower magnifications. The contrast of the normal photomicrograph is insufficient, especially for comparisons of the lower stages of degeneration. To overcome this trouble and to produce a greater degree of contrast, it was found necessary to transpose the negatives, which decreased the detail of the undegenerated structure and increased that of the degenerated tracts.

Figs 3-8 show the six stages of protection against degeneration, (a) in each case, shows the entire spinal cord

($\times 14$) and (b) the corresponding anterolateral column ($\times 45$), where the degeneration on which the classification was based chiefly occurred.

It is evident from these photomicrographs that the first degeneration in vitamin A deficiency occurs in the funiculus praedorsalis and develops gradually in the columns of Goll and Burdach. Even in the second stage of protection the posterior columns are affected to only a very slight degree in comparison with the degeneration in the anterolateral columns. However, the severity of degeneration in the posterior columns increases rapidly in the later stages and reaches the same degree as in the anterior columns when there is no protection.

Studies in Detoxication

28 THE BIOSYNTHESIS OF *o*, *m*- AND *p* CYANOPHENYLGLUCURONIDES

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(Received 3 August 1949)

Studies in progress in this laboratory on the fate of cyanobenzene in the rabbit (Smith & Williams, 1949) required as reference compounds the glucuronides of the *o*, *m* and *p* cyanophenols. These compounds were made biosynthetically by feeding the necessary phenols to rabbits and isolating the glucuronides from the urine. Only *p* cyanophenylglucuronide was isolated as such, the *o* and *m* glucuronides being obtained as crystalline amides and triacetyl methyl esters.

EXPERIMENTAL

o Cyanophenylglucuronide

o Cyanophenol (1.5 g, *m.p.* 90°)* was fed in aqueous suspension to a 3.5 kg rabbit. No ill effects were noted, and after 4 hr the urine (70 ml) was collected. The urine gave negative tests with Benedict reagent, FeCl₃ and dichloroquinonechloroimide, but an intense test with naphthoresorcinol. The basic Pb acetate precipitate of the urine was prepared as described in earlier papers (e.g. Smith & Williams, 1948). The Pb was removed with H₂S and the aqueous residue taken to a gum *in vacuo*. This gum (*o* cyanophenylglucuronide) was soluble in 95% ethanol and water, but attempts to crystallize it or to form crystalline salts with benzylamine or triethanolamine were not successful. Urine collected after 4 hr contained only traces of the glucuronide. The gum was dissolved in 5 ml 95% ethanol and methylated by shaking for an hour with an excess of diazomethane in 150 ml diethyl ether. The mixture was filtered and the ether removed *in vacuo* leaving a neutral gum which was presumably the methyl ester of *o* cyanophenylglucuronide. This ester was now dissolved in a mixture of 10 ml. acetic anhydride and 10 ml. pyridine. After 20 hr at room temperature, the mixture was diluted to 100 ml with water and cooled to 0°. Crystals (1.35 g or 25% of the dose) of the methyl ester of triacetyl β *o*-cyanophenyl-*D*-glucuronide were thrown out. These were collected and recrystallized from absolute ethanol ligroin as long, colourless needles, *m.p.* 151–152° and $[\alpha]_D^{20} -71^\circ$ (*c.* 4.9 in CHCl₃) (Found C, 55.46, H, 4.8, N, 3.4, OMe, 7.2. C₂₅H₂₁O₁₀N requires C, 55.2, H, 4.9, N, 3.2, OMe, 7.1%). The compound was sparingly soluble in hot water, hot ligroin and CCl₄, easily soluble in CHCl₃, acetone, ethanol and ethyl acetate and insoluble in light petroleum.

β *o* Cyanophenyl-*D*-glucuronidamide. The above acetylated ester (0.1 g) was dissolved in 5 ml absolute ethanol, and the solution saturated with NH₃ at 0°. On standing overnight β *o*-cyanophenyl-*D*-glucuronidamide hemihydrate was de-

posited as fine colourless needles. It was recrystallized from ethanol/light petroleum and had *m.p.* 195° and $[\alpha]_D^{20} -78^\circ$ (*c.* 1.3 in 50% aqueous ethanol) (Found C, 51.3, H, 5.0, N, 9.7, C₁₅H₁₄O₆N. 0.5 H₂O requires C, 51.5, H, 5.0, N, 9.5%). The compound was soluble in water, sparingly soluble in ethanol and insoluble in ether and light petroleum.

m Cyanophenylglucuronide

m Cyanophenol (2 g, *m.p.* 83°) was fed, as a suspension in water, to a 3 kg rabbit and the clear yellow 4 hr urine (100 ml), which gave only the naphthoresorcinol test, was worked up as for the *o*-compound. *m* Cyanophenylglucuronide was obtained as a gelatinous solid which dried to a non crystalline powder soluble in 95% ethanol and water. The powder was methylated in ethanol for 3 hr with excess ethereal diazomethane to give a gummy methyl ester. This ester was acetylated as above and yielded 2.9 g (40% of dose) of the crystalline methyl ester of triacetyl β *m* cyanophenyl-*D*-glucuronide. Its solubility was similar to that of the *o* compound. On recrystallization from absolute ethanol the compound formed colourless needles, *m.p.* 156–157° and $[\alpha]_D^{20} -37^\circ$ (*c.* 3.2 in CHCl₃) (Found C, 55.3, H, 5.2, N, 3.7, OCH₃, 7.2. C₂₀H₁₄O₁₀N requires C, 55.2, H, 4.9, N, 3.2, OCH₃, 7.1%).

β *m* Cyanophenyl-*D*-glucuronidamide was obtained by treating the above ester (0.25 g) in ethanol (4 ml.) with NH₃, and after keeping the solution overnight the amide (0.1 g) was precipitated from the solution by ether. On recrystallization from hot ethanol, the amide formed colourless needles of the monohydrate, *m.p.* 207° (decomp) and $[\alpha]_D^{25} -63^\circ$ (*c.* 0.4 in water) (Found C, 50.4, H, 5.3, N, 9.5. C₁₅H₁₄O₆N₂ · H₂O requires C, 50.0, H, 5.2, N, 9.0%). It was soluble in water and ethanol but not in benzene or ether.

p Cyanophenylglucuronide

p Cyanophenol (1.5 g, *m.p.* 110°) was fed to a 3 kg rabbit as before. A 4 hr urine (100 ml) was collected which gave no colour with FeCl₃, no reduction with Benedict reagent but an intense naphthoresorcinol test. On extracting the untreated urine with ether, an extract was obtained which gave feebly, with FeCl₃, the colours characteristic of a catechol derivative. The glucuronide fraction of the urine was separated by the Pb acetate procedure and the Pb free filtrate was taken down *in vacuo* to a thin syrup, which solidified on standing. The solid was treated with a little water and the crystals (230 mg or 6% of the dose) of *p* cyanophenylglucuronide monohydrate filtered off. The compound on recrystallization from water formed colourless needles, *m.p.* 140° (decomp) and $[\alpha]_D^{20} -92^\circ$ (*c.* 3.5 in

* All melting points are uncorrected.

Table 1 *Melting points and optical rotations of biosynthetic cyanophenylglucuronides and derivatives*

	Glucuronide		Triacetyl methyl ester of glucuronide		Glucuronamide	
	M.p	$[\alpha]_D^{20}$ in water	M p	$[\alpha]_D^{20}$ in CHCl_3	M.p	$[\alpha]_D^{20}$ in water
Cyanophenol						
ortho	Not crystallized		151°	-71°	195°*	-78°†
meta	Not crystallized		156-157°	-37°	207°†	-63°
para	140°‡	-92°	130-135°	-42°	210-213°	-72°

* Hemihydrate † In 50% aqueous ethanol ‡ Monohydrates

water) (Found C, 50.15, H, 4.9, N, 4.45 $\text{C}_{12}\text{H}_{13}\text{O}_7\text{N}$, H_2O requires C, 49.85, H, 4.8, N, 4.5%) The glucuronide was soluble to the extent of 5% in water, it was more soluble in ethanol but insoluble in ether.

The mother liquors after the separation of the crystalline glucuronide were evaporated to dryness, and the residue dissolved in a little 95% ethanol. The solution, on treatment with ethereal diazomethane as before, gave a gummy methyl ester. The latter, on acetylation with pyridine and acetic anhydride for 48 hr at room temperature and then pouring into water, yielded the crystalline methyl ester of triacetyl β *p* cyanophenyl β glucuronide (0.51 g or 9% of the dose). On recrystallization from ethanol light petroleum the compound formed colourless needles, m.p. 130-135° and $[\alpha]_D^{20} -42^\circ$ (c, 1.4 in CHCl_3) (Found C, 54.8, H, 4.75, N, 3.65 $\text{C}_{20}\text{H}_{21}\text{O}_{10}\text{N}$ requires C, 55.2, H, 4.9, N, 3.2%).

β *p* Cyanophenyl- β -glucuronidamide. Treatment of the above acetylated ester (0.3 g) with ethanol saturated with NH_3 at 0°, yielded after keeping overnight no crystalline material. However, on diluting the solution with light petroleum, the amide was thrown out as an oil which gradually solidified (yield 0.15 g). It was recrystallized from ethanol benzene and formed fine colourless needles, m.p. 210-213° (decomp) and $[\alpha]_D^{20} -72^\circ$ (c, 2.6 in water) (Found C, 53.3, H, 5.1, N, 9.5 $\text{C}_{12}\text{H}_{14}\text{O}_8\text{N}_2$ requires C, 53.05, H, 4.8, N, 9.5%).

DISCUSSION

The properties of the *o*, *m* and *p* cyanophenylglucuronides and their derivatives are summarized in Table 1.

The only figure calling for comment is the specific optical rotation of the methyl ester of triacetyl

o cyanophenylglucuronide which is nearly twice that of its isomers. The rotations of the three amides are, however, of the same order. It is known that the tetra acetates of some *ortho* substituted phenyl β glucosides have anomalous rotations (see Pigman & Goepp, 1948) when compared with the *m* and *p* isomers. In view of this, the high negative rotation of triacetyl *o* cyanophenyl β glucuronide methyl ester compared with the *m* and *p* isomers is not unexpected. It may be noted also that the methyl ester of triacetyl β *o* methoxyphenylglucuronide (Garton & Williams, 1948, 1949) has $[M]_D -21,560$ in ethanol, while the corresponding derivatives of β *m* acetoxyphephenyl- and β *p* acetoxyphephenyl glucuronides have $[M]_D$ values about half of this ($-11,466$ and $-10,296$, respectively) in acetone.

One further point in this work is worthy of comment and that is that the main bulk of the cyanophenylglucuronides was excreted within 4 hr of administering the cyanophenols, none of which was toxic at 0.5 g/kg.

SUMMARY

- 1 The preparation of *p* cyanophenylglucuronide, by feeding *p* cyanophenol to rabbits, is described.
- 2 The characterization of the biosynthetic *o*, *m* and *p* cyanophenylglucuronides as their amides and triacetyl methyl esters, is described.

The author wishes to thank Prof R. T. Williams for his stimulating interest and encouragement in this work.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 280th Meeting of the Biochemical Society was held in the Biochemistry Department, King's College, Strand, London, W O 2, on Saturday 22 October 1949, when the following papers were read

COMMUNICATIONS

Preformed Vitamin A in Northern Krill By S K KON and S Y THOMPSON (National Institute for Research in Dairying, University of Reading)

Wagner (1939) claims to have extracted 14.5 mg of pure β carotene from 1 kg of Crustacea ('Gattung der Euphausia superba Dana') forming the food of Blue and Fin whales caught at the Lofra whaling station in the Faeroes. It is generally accepted that astaxanthin pigments are the main carotenoids of Crustacea and that β carotene is present, if at all, only in small amounts. Blue and Fin whales live in northern waters largely on two euphausiids *Meganyctiphanes norvegica* and *Thysanoessa inermis* (cf. Einarsson, 1945). With the generous help of the Marine Station at Millport, Isle of Cumbrae, we obtained specimens of *Meganyctiphanes norvegica*, of *Thysanoessa raschi*, closely allied to *T. inermis*, and of several other species of Crustacea all from Loch Fyne. A further mixed sample of *Meganyctiphanes norvegica* with small plankton and a sample of the common shrimp, *Crangon vulgaris*, were kindly supplied by the Marine Biological Laboratory at Aberdeen.

mostly in the ester form, were found in *Meganyctiphanes norvegica* from Loch Fyne and in *Thysanoessa raschi*. The vitamin A fraction gave a typical Carr Price reaction, and the potency calculated on this basis agreed with that obtained from the ultra-violet absorption curve corrected for irrelevant absorption. The fraction was further identified by its yellow fluorescence in ultraviolet light and by mixed chromatography of the original extract with vitamin A ester and, after saponification, with vitamin A alcohol.

Our observations with *Meganyctiphanes norvegica* agree with those already reported on *Euphausia superba* Dana, the Krill of antarctic whales (Thompson *et al.* 1949, Kon & Thompson, 1949), and indicate that, contrary to Wagner's (1939) claims, those whales feeding on larger Crustacea derive their vitamin A from preformed vitamin A and not from carotene. A whale's stomach may contain up to 1200 l of Krill (Einarsson, 1945), and

Species	Locality	Carotenoids				
		Vitamin A		Total*		β Carotene
		$\mu\text{g/g}$ shrimp	$\mu\text{g/g}$ oil	$\mu\text{g/g}$ shrimp	$\mu\text{g/g}$ oil	
<i>Meganyctiphanes norvegica</i>	Loch Fyne	15	680	42	1900	Faint trace
<i>Thysanoessa raschi</i>	Loch Fyne	32	495	33	500	Faint trace
<i>Pandalus bonnier</i>	Loch Fyne	2.1	89	24	1000	Faint trace
<i>Spirontocaris spinus</i>	Loch Fyne	1.0	22	27	950	Trace
<i>Crangon allmanni</i>	Loch Fyne	0.4	30	5	390	Trace
<i>Meganyctiphanes norvegica</i> , mixed sample with other plankton	Neighbourhood of the Faeroes	1.6	145	23	2100	Faint trace
<i>Crangon vulgaris</i>	Bay of Holland, Stronsay, Orkneys	0.2	21	5	550	Trace

* Read at the absorption maximum for β carotene and calculated as such.

The Crustacea were identified and placed in absolute alcohol immediately after netting. After extraction of the fat with light petroleum and saponification, chromatographic separation and measurement of vitamin A and β carotene were done as described by Thompson (1949) and Thompson, Ganguly & Kon (1949).

None of these Crustacea contained more than traces of β carotene, but, as shown in the table, relatively large quantities of preformed vitamin A,

such enormous quantities could, no doubt, account for the richness of whale liver in vitamin A. The origin of these stores has so far been in doubt because of conflicting evidence about the presence of vitamin A and active carotenoids in zooplankton.

Our grateful thanks are due to Dr E. Ford for all the facilities at Millport, to Dr A. P. Orr for his generous and kind help there in the collection of specimens, to Dr C. E. Lucas for samples and to Dr J. A. Lovern for constant interest and advice.

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Naturally Occurring Organo-silicon Compounds By P F HOLT and DEIRDRE M YATES
(University of Reading)

The separation of an alcohol soluble fraction of plasma in which were silicon containing compounds was described by Holzapfel (1943) Ohlmeyer & Olpp (1944) described a procedure for the determination of the organic silicon compounds in tissues which involves their extraction from the tissues by ether alcohol, ignition and fusion of the extract with sodium carbonate and the colorimetric determination of the silicon

In attempting to fractionate the alcohol extract of liver and egg, no agreement could be obtained between the quantity of silicon in the alcoholic extract and the sum of the quantities in the fractions It would appear that, using the method of fusion in an open crucible, a loss is incurred because of the volatility of the organo silicon compounds By applying the technique of the Parr bomb, combustion is effected without loss of volatile substances Values obtained on an extract of egg yolk were higher and much more consistent than those obtained by the method of Ohlmeyer & Olpp

Method Water is abstracted from the tissue by the Dean and Stark procedure (Warne, 1942), their apparatus being modified to handle larger quantities of material

The dry tissue is extracted under reflux with ethanol for 10–20 hr (Experiments on chicken liver indicated that extraction is complete after about 6 hr) The ethanol solution is filtered and the filtrate is evaporated to a brown fatty residue Approximately 500 mg of this residue is trans

ferred to a nickel Parr micro bomb, the crucible of which is half filled from a mixture of 0.2 g finely powdered sucrose and 6 g sodium peroxide The mixture is pressed hard into the bomb which is then closed and ignited, applying a small flame from an oxygen coal gas hand blowpipe to the base of the crucible The bomb is cooled, unsealed and dropped into water in a plastic beaker The silica is determined colorimetrically in an aliquot of the resulting solution after separation of phosphate by the method of King (1939)

Results Since the identity of the silicon compounds is unknown, it is not possible to determine the accuracy of the method by making known additions to a tissue extract and comparing the recovery values However, a number of estimations has been made on tissue extracts by both the bomb method and that of fusion in an open crucible Whilst the former method gives reasonably consistent values, the latter gives figures invariably lower than those obtained with the bomb and extremely variable Examples of figures obtained with an extract of egg yolk are given in Table 1

Table 1 *Silica content of extract of egg yolk*
(mg SiO₂ per 100 g extract)

Alkaline fusion	Bomb method
16.1	29.4
11.1	30.7
1.9	30.1
14.0	29.8
7.5	37.1
17.4	32.3

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The Quantitative Analysis of Ribonucleic Acids By R MARKHAM and J D SMITH (Agricultural Research Council Plant Virus Research Unit, Moltano Institute, Cambridge)

The recently developed methods of analysis of purines and pyrimidines by paper partition chromatography (Hotchkiss, 1948, Vischer & Chargaff, 1948, Markham & Smith, 1949) have made it possible to detect and estimate minute quantities

of the free bases and their ribosides The application of these techniques to the analysis of ribonucleic acids has, on the other hand, been complicated by the lack of a satisfactory method of hydrolysis for freeing the pyrimidines or their nucleosides quanti-

tatively, and little reliance can be placed on published values for the pyrimidine content of these substances. On the other hand, hydrolysis by N hydrochloric acid at 100° for 1 hr releases purines as such and the pyrimidines as nucleotides quantitatively. We have found it possible to resolve this mixture in several ways using two dimensional chromatography or several runs in different solvents, but, with a view to simplifying the

method as much as possible, we have investigated the effects of the alteration of water content and acidity on solvent systems based on the simple alcohols. Using this information we have designed a solvent which will resolve guanine, adenine, cytidylic acid and uridylic acid on a single dimensional chromatogram. With this new technique it is possible to make a complete quantitative base analysis on as little as 0.1 mg of ribonucleic acid.

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The Absorption of Vitamin A. By EVA EDEN and K. C. SELLERS (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council, and the Veterinary Investigation Centre, Institute of Animal Pathology, Cambridge*)

The absorption of vitamin A by the lymph stream has been demonstrated in several species (see previous communication, Eden & Sellers, 1949). Earlier studies on the mechanism of vitamin A absorption by Gray, Morgareidge & Cawley (1940) showed that in the rat vitamin A esters were hydrolysed in the intestinal tract. To our knowledge, the changes of the form of vitamin A during absorption have not been examined in ruminants, hence it was decided to repeat and extend the work of Gray *et al.* (1940), using sheep and bovines of varying ages as experimental animals.

Twenty animals were dosed orally with vitamin A alcohol or ester (Distillation Products Ltd.), emulsified in reconstituted separated milk. The dose of vitamin A was adjusted to 5000 i.u./kg. of body weight and the animals were slaughtered 4 hr. after dosing. This was the time interval chosen, as previous experiments (Eden & Sellers, 1949) had shown that, although the rate of absorption of vitamin A was variable, it usually reached its maximum at this time. The concentrations of vitamin A alcohol and ester were estimated in the blood before and after dosing. Estimations were also made on slaughter in the intestinal contents

and wall and in the lymph. Vitamin A alcohol and ester were separated chromatographically using a modified method of Hoch & Hoch (1946). (Further details of the technical procedure will be published later.)

Four hours after the administration of vitamin A esters almost complete hydrolysis had occurred in the intestinal lumen of some bovines, whereas in others hydrolysis was only partial. Estimations performed on the intestinal mucosa at the same time showed that about 75% of the absorbed vitamin A was in the ester form, and the same was true in animals dosed with vitamin A alcohol. These results indicate that the intestinal wall is capable of esterifying free vitamin A alcohol, but so far we have been unable to obtain evidence that esterified vitamin A is absorbed in its unaltered form. After leaving the intestinal wall, the absorbed vitamin A apparently remained esterified, as the rise of the vitamin in the intestinal lymph following administration of either form was mainly present in the ester form. Confirmatory results have also been obtained in experiments performed on sheep and newborn calves.

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Further Studies of the Biogenesis of Porphyrins in Rabbits By HELEN M. MUIR and A. NEUBERGER (*National Institute for Medical Research, Hampstead, N.W. 3*)

The specific incorporation of the nitrogen of ^{15}N -labelled glycine into haemin has been clearly demonstrated by Shemin & Rittenberg (1946), and it has also been shown that the isotope is equally distributed between both types of pyrrole ring (Muir & Neuberger, 1949, Wittenberg & Shemin, 1949). Of the two carbon atoms of glycine only the methylene carbon atom appears in the porphyrin molecule (Grinstein, Kamen & Moore, 1948, Altmann, Casarett, Masters, Noonan & Salomon, 1948). Rittenberg has also identified acetate as a precursor of porphyrins (Bloch & Rittenberg, 1945, Radin, Rittenberg & Shemin, 1949).

In the present investigation rabbits were injected with the following three isotopic compounds



The blood porphyrin was isolated and degraded by oxidation by methods previously described (Muir & Neuberger, 1949). The relative activities of the three

activities of the carbonate fraction indicate clearly that most if not all the methyne bridge carbon atoms are derived from the original glycine. The $^{14}\text{C}/^{15}\text{N}$ ratio in the porphyrin, as compared with that of the glycine, showed that there are about 2.4 times as many carbon atoms derived from glycine as nitrogen atoms. If it is assumed that the four N atoms of porphyrin come from glycine and that no extensive reversible deamination of glycine takes place, it follows that all four methyne carbon atoms and at least as many of the ring carbon atoms are derived from the methylene group of glycine.

In experiments using isotopic acetates, the utilization of the methyl group of methyl-labelled acetate is found to be considerably more efficient than the utilization of the carboxyl group of carboxyl-labelled acetate. Furthermore, the distribution of the carbon atoms from these two precursors is not the same. In the case of methyl-labelled acetate, both kinds of pyrrole ring are

Table 1

Specific activities (counts/min / μ mole)

Substance administered	Mesoporphyrin	Haematinic acid	Imide	BaCO ₃
$^{15}\text{NH}_2, ^{14}\text{CH}_2\text{COOH}$	107.5 \pm 1.75	17.52 \pm 0.27	17.39 \pm 0.23	9.9 \pm 0.3
$\text{CH}_3, ^{14}\text{COOH}$	32.35 \pm 1.09	12.06 \pm 0.31	2.97 \pm 0.15	0.44 \pm 0.33
$^{14}\text{CH}_3\text{COOH}$	27.9 \pm 0.67	7.55 \pm 0.25	6.5 \pm 0.22	0.08

fragments methylethylmaleic acid imide, derived from rings I and II, haematinic acid from rings III and IV and CO₂ mainly derived from the methyne carbon atoms are shown in Table 1. It can be seen that with both the acetates the two imides account for the whole of the activity of the mesoporphyrin, but with glycine only 65%. This and the relative

equally radioactive, but after injecting carboxyl-labelled acetate, the acidic pyrroles from rings III and IV are considerably more radioactive than those from rings I and II, which are not acidic. It was also possible to exclude both the acetates from being direct precursors of the methyne carbon atoms. The implications of these results were discussed.

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A Modified Chemical Estimation of Nicotinic Acid By D. E. HUGHES (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield 10*)

Nicotinic acid reacts with cyanogen bromide to form a yellow coloured derivative of glutaconic aldehyde (König, 1904), which can be estimated photometrically either directly (Kokovichyna, 1946) or after condensation with various amines (Harris & Raymond, 1939). It was found that neither method

was satisfactory for estimating accurately small quantities of nicotinic acid (10–25 μmol). This was due to low light absorption of the aldehyde or to instability of the amine derivatives.

Treatment of the glutaconic aldehyde derivative with NaOH resulted in the formation of material

with a strong absorption in the near ultra violet (compare Larsen & Haag (1944) and Chaudhuri & Kodicek (1949)) The peak of the absorption curve was at $372\text{ m}\mu$. The $\epsilon_{372\text{ m}\mu}^{\text{max}} = 3.02 \times 10^4$ (maximum molar extinction coefficient on the basis of nicotinic acid) The absorption obeyed Beers's law with quantities of nicotinic acid ranging from 0.625 to 100 μmol in a total volume of 3.0 ml

The method of estimation was as follows fresh CNBr (Kodicek, 1940) is diluted with an equal quantity of buffer (100 ml 0.5 M phosphate buffer pH 6.0, and 100 ml 0.5 M acetate buffer pH 5.0) The pH of the solution is adjusted to 5.5–5.6 with N NaOH or 10% acetic acid and checked with a glass electrode The pH of the unknown nicotinic acid solution, as adjusted to pH 5.3–5.8 and 0.5 ml is added to 1 ml of the CNBr reagent The mixture is heated at 80° for 20 min and then cooled Next are added 0.5 ml of 6 N NaOH and 1.0 ml of water The solution is left for 45 min in the dark at room temperature, and the absorption is measured in the Beckmann spectrophotometer (model DU) The

readings increase some 10% in the next 18 hr after which they reach a maximum value Duplicate samples with added nicotinic acid are run at the same time to eliminate the effect of salt concentration and pH

Nicotinamide, nicotinamide ribose phosphate and cozymase gave an absorption equal to 16.5% of that of nicotinic acid at $372\text{ m}\mu$, but the peak was at $352\text{ m}\mu$ ($\epsilon_{352\text{ m}\mu} = 1.16 \times 10^4$) Aneurin gave an absorption peak at $372\text{ m}\mu$ ($\epsilon_{372\text{ m}\mu} = 0.92 \times 10^4$) Nicotinyl hydrazide and nicotinuric acid behaved as nicotinic acid while trigonelline, nicotinamide N^1 methylchloride, pyridoxine, pyridoxal and pyridoxamine did not absorb between 400 and $340\text{ m}\mu$

The method was used to estimate nicotinic acid in solutions containing glucose and salts and in acid hydrolysates of *Lactobacillus arabinosus* The results were in good agreement with those of the microbiological method The method also promises to be useful for estimating separately nicotinamide and nicotinic acid in solutions containing both

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An Improved Medium for Microbiological Assays with *Lactobacillus casei* By E. KODICEK and S. P. MISTRY (Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council)

The requirements of *Lactobacillus casei* for the individual constituents present in synthetic media have been studied and a modified medium* has been devised which can be used for a number of vitamin assays including pteroylglutamic acid, riboflavin, nicotinic acid, biotin, pantothenic acid, streptogenin and also for the estimation of tryptophan

The medium has been developed specifically for the estimation of pteroylglutamic acid. It was found that an overall increase in the concentration of B vitamins was essential for obtaining a maximum

acid production of 28–30 ml 0.1 N acid per 10 ml of basal medium which is almost equal to the theoretical yield of acid which could be produced from 3% glucose. A new salt mixture has been added and 3% potassium acetate was used instead of the sodium salt. The medium has been employed for routine assays of nicotinic acid and of streptogenin (Kodicek & Mistry, 1949). The estimation of nicotinic acid using *L. casei* was found to give more reproducible results than were obtained with the previous method employing *L. arabinosus* (Kodicek & Pepper, 1948). Only solutions of the other crystalline vitamins have been tried and extracts have not been assayed so far.

* Composition of basal medium, per 10 ml: glucose and potassium acetate (hydrated), 300 mg each; K_2HPO_4 and KH_2PO_4 , 25 mg each; salt solution E (Kodicek & Pepper, 1948) 0.05 ml; 'Labco' vitamin free casein, H_2SO_4 hydrolysed, 50 mg; norite treated 'Difco' peptone, 2 mg; DL-tryptophan and L-cystine, 2 mg each; DL-alanine, 1 mg; adenine, guanine, uracil and xanthine, 0.1 mg each; aneurine, riboflavin, nicotinic acid and calcium pantothenate, 10 μg each; pyridoxine, 25 μg ; p-aminobenzoic acid, 2 μg ; pteroylglutamic acid, 50 μg ; biotin (free acid), 40 μg . (Norite treated peptone omitted for biotin, streptogenin and tryptophan assays; L-Asparagine, 1 mg, included for streptogenin assay.)

The new medium has been compared with media used by other workers and has been found to give more satisfactory results. It contains acid hydrolysed casein, which is a more reproducible constituent than the enzymatic digest used by Roberts & Snell (1946). Furthermore, when assaying streptogenin activity, enzymatic digests of casein could not be used because of their streptogenin content and so far no methods are available to free them completely of this factor.

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Cozymase Synthesis by *Lactobacillus arabinosus* 17-5 By D E HUGHES and D H WILLIAMSON
 (Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield 10)

The rate of anaerobic glycolysis by washed suspensions of nicotinic acid deficient *Lactobacillus arabinosus* is increased from two to fourfold by the addition of nicotinic acid, nicotinamide or cozymase (McIlwain, 1949). The acceleration of glycolysis is due to the increase of cellular cozymase (McIlwain, Stanley & Hughes, 1949) which is relatively stable in this organism (McIlwain & Hughes, 1948). For these reasons *Lb arabinosus* has been used in the following preliminary studies on the bacterial synthesis of the cozymase. Cozymase and nicotinic acid were estimated by the methods previously outlined (McIlwain & Hughes, 1948). Glycolysis by washed suspensions of cells was followed manometrically. Cozymase synthesis was negligible in the absence of glucose.

The maximum rate of synthesis occurred in cells harvested after 16-18 hr growth on the medium of Barton Wright (1946), deficient in nicotinic acid (1.67×10^{-7} M). Cells harvested earlier were not deficient and cells harvested later were deficient but synthesized cozymase more slowly. With cells grown for longer than 18 hr the rate of glycolysis also falls but not to such a great extent as cozymase synthesis. The addition of 10^{-6} M nicotinic acid to old cells (44 hr growth) resulted in a maximum stimulation of glycolysis of 300% after a lag period of 40 min. If nicotinic acid was replaced by equimolar amounts of nicotinamide or cozymase

the lag period before the attainment of maximum glycolysis was 10-15 min. It would seem therefore that the system responsible for amidation of nicotinic acid has decayed at a faster rate than other systems involved in the synthesis of cozymase.

At nicotinic acid concentrations below 4×10^{-5} M the rate of cozymase synthesis was reduced. At higher concentrations the maximal rate of synthesis was maintained for only the first 10-15 min and then fell progressively until the cellular concentration of coenzyme reached an average of 12.0 μ mol/mg dry wt cells. The final rate of synthesis was from 1.5 to 3.0 μ mol/mg dry wt/hr which was sufficient to replace a cozymase like substance lost from the cells. None of the constituents of the growth medium affected either the rate of synthesis or the level of cozymase in the cells. Cells grown with adequate amounts of nicotinic acid (2×10^{-4} M) synthesized cozymase from nicotinic acid at a low rate (< 3.0 μ mol/mg dry wt/hr) compared with cells grown on the nicotinic acid deficient medium whose maximum rate of synthesis 24 μ mol/mg dry wt/hr. Since these organisms do not break down cozymase at a rate comparable to the rate of synthesis (see McIlwain & Hughes, 1948) both the rate of synthesis and the level of cozymase in the cell appears to be controlled by a mechanism which inhibits synthesis as the cells approach saturation with cozymase.

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Inactivation of Serum Alkaline Phosphatase by Adrenaline and Related Substances By A B ANDERSON (Biochemical Laboratory, North Middlesex Hospital, London, N 18)

Using sodium β glycerophosphate as substrate in glycine buffer pH 9.4 (Jenner & Kay, 1932) and serum of high alkaline phosphatase content as the source of enzyme, it was found that adrenaline in a concentration of 5×10^{-4} M inhibited the phosphatase to the extent of 70-80%. Adrenochrome in a

concentration of 2.5×10^{-4} M gave 100% inhibition. An equivalent inhibition, i.e. 70%, was found with strong oxidizing agents such as potassium permanganate, as has been reported by Sizer (1942). Of the other phenolic compounds investigated only pyrogallol gave an equivalent inhibition of 70% at

at a concentration of $5 \times 10^{-4} M$. Slight inhibition was found with catechol 9%, quinhydrone and quinol 13% and *p*-quinone 22% at this concentration, while tyrosine, tyramine, resorcinol and ephedrine did not inhibit at all.

In view of the reported dependence of phosphatase activity on oxidation reduction potential

(Sizer, 1942) measurements of the E_h of the complete reaction mixtures were made immediately after the addition of the inhibitors and again after 30 min incubation. No correlation was found between E_h and degree of inhibition.

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The Inhibition of Plant Phosphorylases by β -Amylase and the Detection of Phosphorylase in Barley By HELEN K PORTER (*Research Institute of Plant Physiology, Imperial College of Science and Technology, London, S W 7*)

Extracts (1 ml H_2O to 1 g ground grain) of ungerminated barley (BE) slowly convert glucose 1-phosphate (G1P), after a lag of about 4 hr, to an ester resistant to hydrolysis in 7 min by α -acid. Very small amounts of free phosphate (P) are simultaneously released. When either potato or broad bean phosphorylase is included in a 1 ml digest at pH 6.8 containing 0.1 ml BE, 0.035 M-G1P and 200 mg % soluble starch (s.p.) no P is released in the 1-2 hr required for the reaction yielding amylose to reach equilibrium in the absence of BE. This inhibition is reduced and finally eliminated by continuous dilution of BE, and is removed by heat. Crystalline β -amylase behaves in the same way as BE towards added phosphorylase indicating that β -amylase (β) is the inhibiting agent in BE. Under the same conditions wheat, oat and rye extracts inhibit the bean enzyme to the extent of 70-80%.

If s.p. is exposed to the action of BE, its capacity to prime the phosphorylase reaction, measured by the amount of P released in 2 hr following inactivation of β by heat, is reduced by 25% after 90 min, and by 40% after 24 hr. In the unheated containing potato or bean enzyme there is no primer present for many hours, and if

these enzymes were operative, release of P should be observed. It is concluded that β is inhibiting phosphorylase action and by some mechanism other than primer destruction. This view is supported by experiments using amylose glycolate, pretreated with β (AG), instead of s.p. Here potato enzyme effects release of only traces of P in 44 hr, although the priming capacity of AG is unimpaired as shown by subsequent heat inactivation of BE and further addition of potato enzyme.

Phosphorylase has been detected in BE by employing $(NH_4)_2SO_4$ to lower the ratio of β to phosphorylase to a level where inhibition is only partial. Throughout extraction and fractionation pH is maintained at 6.8 to avoid phosphorylase loss. Three fractionations of the precipitate formed between 40 and 50% saturation yield a preparation which liberates P from G1P relatively rapidly, and which with an achroic primer forms a polysaccharide giving a weak blue stain with iodine, after 5-10 min. This iodine staining material persists in the digest for 20-30 min. Finally, addition of starch and P to the preparation leads to formation of G1P.

The gift of samples of AG and crystalline β -amylase by Prof. S. Peat and Prof. A. K. Balls is gratefully acknowledged.

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

- The swelling of collagen in alkaline solutions 1 Swelling in solutions of sodium hydroxide By JOANE H BOWEN and R H KENTEN
- A study of the peptides of cystine in partial hydrolysates of wool By R CONSDEN and A H GORDON
- The respiration of rat-liver slices in the presence of some aliphatic amines, hydroxy amines and quaternary ammonium salts By C LONG
- The reaction of oxidizing agents with wool 1 The division of cystine into two fractions of widely differing reactivities By P ALEXANDER, R F HUDSON and M FOX
- The utilization of non protein nitrogen in the bovine rumen 5 The isolation and nutritive value of a preparation of dried rumen bacteria By M L McNAUGHT, J A B SMITH, K. M HENRY and S K KONG
- The utilization of non protein nitrogen in the bovine rumen 6 The effect of metals on the activity of the rumen bacteria By M L McNAUGHT, E C OWEN and J A B SMITH
- The turnover of radioactive phosphate injected into the subarachnoid space of the brain of the rat By O LINDBERG and L ERNSTER
- The determination of *N*-methyl 2 pyridone 5 carboxylic acid in human urine By W I M HOLMAN and D J DE LANGE
- Studies in the biochemistry of micro organisms 81 The colouring matters of *Penicillium islandicum* Sopp Part 2 Chrysophanic acid, 4,5 dihydroxy 2 methylanthraquinone By B H HOWARD and H RAISTRICK
- Changes in water and ion metabolism and in kidney functions during the development of oedema in rats fed on protein-deficient diets By S E DICKER
- Pigments derived from tryptophan (1) urochrome, (2) tryptochrome By W R FEARON and W A BOGGUST
- The oxidation of manganese by peroxidase systems By R H KENTEN and P J G MANN
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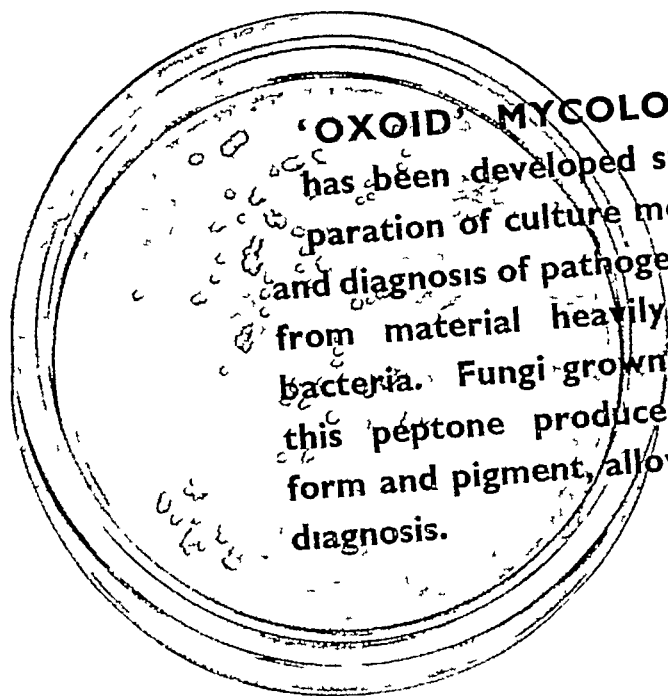
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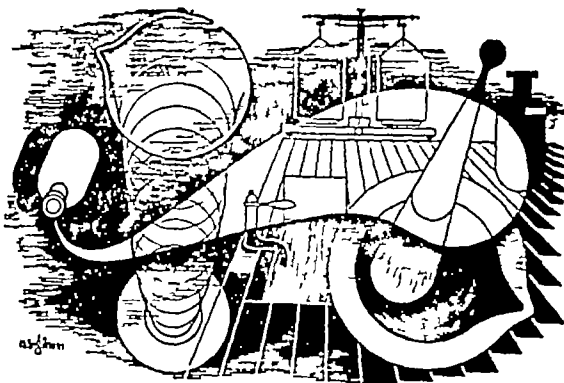
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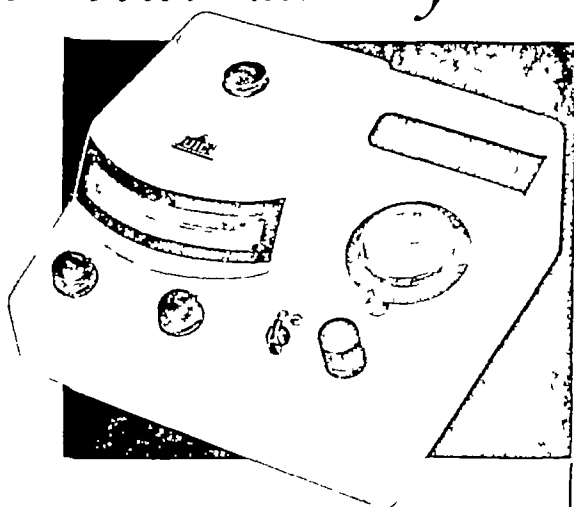
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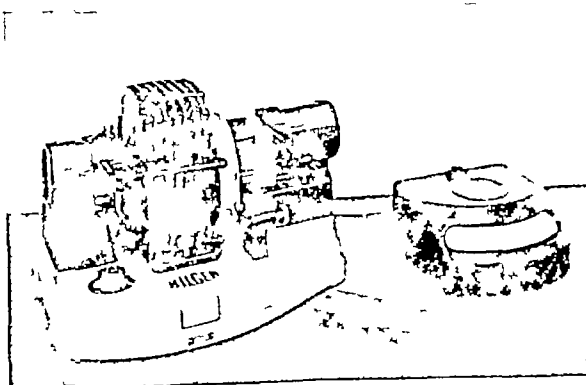
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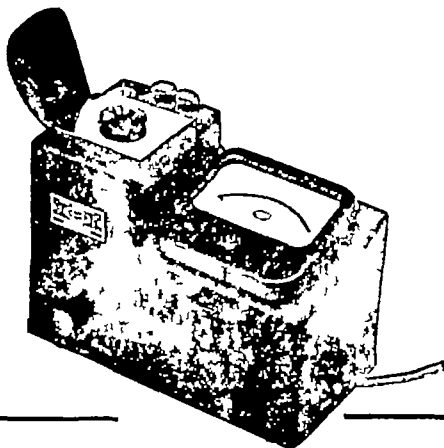
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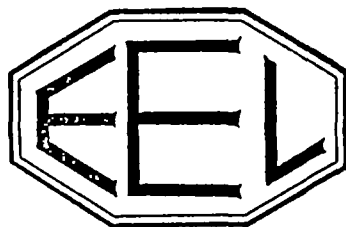


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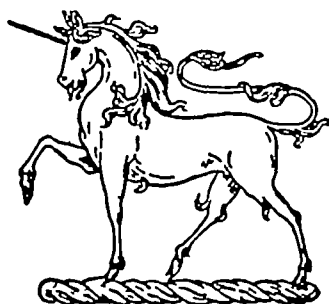
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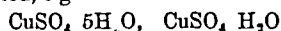
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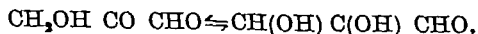
The Intracellular Mode of Action of the Sulphonamide Derivatives Some Condensation Products of Reductone

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(Received 28 March 1949)

Colebrook & Kenny (1936), Colebrook, Buttle & O'Meara (1936) and, later, others showed that some time must elapse after bacteria are inoculated into media containing sulphonamide before an inhibitory effect is produced. Multiplication of the organism occurs in the interval. Subsequently, Wolff & Juhus (1939) concluded that sulphonamides act on bacteria only when they are multiplying, and O'Meara (1942) mentioned that the lethal action of sulphonamides on bacteria is immediately exhibited when the organisms are in the logarithmic phase of growth. This was later confirmed and fully expounded by O'Meara, McNally & Nelson (1947), who pointed out that in the logarithmic phase of growth bacteria are engaged in the utilization of those compounds, present in the culture medium, which are most suited to energy and growth requirements. Glucose is one of the substances. O'Meara, McNally & Nelson (1944) had previously noted that during the logarithmic phase of growth the medium develops strongly reducing properties, and suggested that the reducing substance might be either ascorbic acid, dihydroxyacetone or reductone,



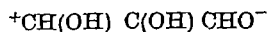
all of which could be produced from glucose. In the earlier publication, O'Meara *et al* (1944) showed that the reducing substance gave a positive 'enediol' [$-\text{C(OH) C(OH)}-$] test with *o* dinitrobenzene and sodium hydroxide (Fearon & Kawerau, 1943). Ascorbic acid is known to be produced in certain bacterial cultures (Busing & Peters, 1940), but this can usually be detected as its dehydro derivative (Fearon & Kawerau, 1943) and the reducing substance produced by the pathogens failed to give the test for dehydroascorbic acid. Reductone is produced when glucose is heated with alkali (von Euler & Klusmann, 1933, von Euler & Martius, 1933) and, since the culture media are alkaline and gave the enediol reaction after heating, O'Meara *et al* (1947) considered that reductone was the most likely substance to be formed in such media.

O'Meara *et al* (1947) showed that reductone is readily condensed with *p* aminobenzoic acid, sulphapyridine, sulphathiazole and sulphanilamide. The reductone used was a crude solution from the

hydrolysis of glucose with sodium hydroxide, nevertheless solid products were obtained in all cases except in the reductone sulphanilamide condensation, where the product was obviously readily soluble in water. The products were hydrolysed in alkaline solution with varying degrees of rapidity, yielding the starting materials. Condensation of the above mentioned amines with ascorbic acid and dihydroxyacetone gave coloured solutions only.

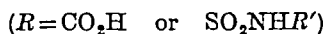
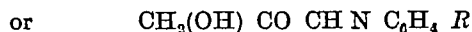
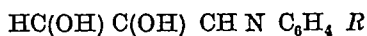
In view of the known biological significance of *p* aminobenzoic acid, and the fact that the compound formed by union of reductone with *p* aminobenzoic acid is readily hydrolysed within the biological range of pH, O'Meara *et al* (1944, 1947) considered that the function of *p* aminobenzoic acid in cellular metabolism is to stabilize and temporarily conserve, for use of the cell, the extremely active substance reductone which can act as a source of energy for the growing cell. Since reductone is readily oxidized (compare von Euler & Martius, 1933) it must be preserved in a stabilized form, but in such a way as to be available for the requirements of the growing cell. It was, in fact, shown by O'Meara *et al* (1947) that *Streptococcus pyogenes* can utilize the reductone *p* aminobenzoic acid condensation product and maintain growth on it, but *p* aminobenzoic acid itself fails to support growth. In extension of the above, it was logical for O'Meara *et al* (1947) to consider that sulphonamides interfere with this source of energy by combination with reductone to form compounds not available for utilization by bacteria. They showed that *Strep. pyogenes* was unable to utilize the sulphapyridine and sulphathiazole derivatives of reductone under conditions identical with those in which the *p* aminobenzoic acid derivative was utilized by these organisms.

Reductone possesses a highly active aldehydic group which readily reacts with electron-donating groups (compare von Euler & Martius, 1933) and reaction may take place by end attack on the charged mesomeric form of the enol



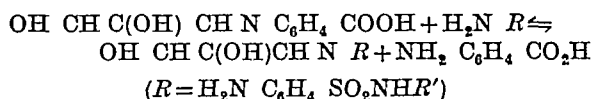
The condensation products of reductone and *p* aminobenzoic acid and the various sulphonamides

are highly coloured, and it is probable that they are of the type



According to the theory propounded by O'Meara *et al* (1947), the effectiveness of the sulphonamides in interfering with the growth of bacteria is linked with the stability of the Schiff bases mentioned above. Those which are most readily hydrolysed will be the least effective in inhibition of growth and *vice versa*, and it is very likely that the ease of hydrolysis of the Schiff bases in the biological range of pH will be dependent upon the solubility of these compounds under these conditions. Before a final conclusion can be reached, the relative solubilities of the compounds must be determined, but it is known that sulphanilamide gives a condensation product with reductone which is very readily soluble in water at neutrality and difficult to isolate, and that sulphanilamide is relatively ineffective as growth inhibitor.

In accordance with the views expressed on the importance of reductone, it seems likely that in mixtures of *p* aminobenzoic acid and a sulphonamide with reductone there will be competition for the aldehydic group by the amino groups. This can probably be represented by the following equation, where only the enolic forms are given,



The position of equilibrium in the above reaction will depend, amongst other factors, upon (a) the affinity of the aromatic amino groups for the aldehydic group, i.e. upon the relative electron donating qualities of these groups, and (b) upon the concentration of the amino compounds. According to Bell & Roblin (1942) and Albert & Goldacre (1942) the basic dissociation constant of the aromatic amino group in the sulphonamides differs only slightly from 2.6×10^{-12} , which is the basic dissociation constant of *p* aminobenzoic acid. Maphenide (marfanil, *p* aminomethylbenzenesulphonamide) is in another category. Here the amino group is aliphatic and the compound correspondingly more basic. In general, it is probable that the concentrations of the amino compounds are important in deciding the position of equilibrium. In the bacterial cell other factors might have prominence such as degree of adsorption of the various compounds on some surface.

In the present investigation our primary object has been the preparation and examination of the products of condensation of reductone with *p* aminobenzoic acid and various sulphonamides. We have

obtained solid products in all cases, but purification, by crystallization, has not always been possible owing to the low solubility of the products in most organic solvents. The reductone sulphanilic acid condensation product is very readily soluble in water, and we have not been able to isolate this compound although its formation undoubtedly occurs, since the colour of the mixed solutions rapidly becomes a deep red.

Although our investigations are not complete we feel that an interim survey is necessary since Angier, Stokstad, Mowat, Hutchings, Boothe, Waller, Semb, Subbarow, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels & Smith (1948) have recently published details of the preparation and properties of the reductone *p* aminobenzoic acid condensation product and some of its derivatives, and Forrest & Walker (1948) have also indicated the scope of their work similar to that which we had contemplated (cf. Bell, Cocker & O'Meara, 1948), and that which has been in hand since September 1947 (cf. O'Meara *et al* 1947).

EXPERIMENTAL

The preparation of reductone Reductone is best prepared by the method of von Euler & Martius (1933) in which glucose is treated with NaOH and the reductone is precipitated as its Pb salt. No improvement in yield was obtained when fructose was employed. The decomposition of the Pb salt with H_2S was, however, performed with shaking in a previously evacuated flask attached to a source of H_2S . This gave a better product than that obtained when H_2S was bubbled through a suspension of the Pb salt (cf. Kuchlin & Böeschen, 1928). The yield of reductone, m.p. 200° , was about 7% of theory. The oxidation of glycerol by FeSO_4 and H_2O_2 (den Otter, 1937) and the oxidation of dihydroxyacetone with copper acetate in ultraviolet light (Kuchlin & Böeschen, 1928) gave disappointing results.

Condensation of reductone with amino compounds The earlier condensations of reductone with the various amino compounds were performed in aqueous sodium acetate, but it was later found that the condensations could be readily performed in aqueous acetone with or without the addition of a few drops of acetic acid. A better product was thus obtained.

Analysis of compounds formed The identity of the compounds was established by analysis and by colorimetric estimation of the *p* aminobenzoic acid or sulphonamide in the compound. This was performed by hydrolysis with dilute alkali, and the resultant amino compound was condensed with *p* dimethylaminobenzaldehyde (Werner, 1939, 1944), the colour so obtained being examined on the Spekker absorptiometer. It was possible approximately to estimate the reductone *p* aminobenzoic acid condensation product by titration with alkali, but the values of the equivalent obtained were variable. When the compound was left in contact with 0.05N NaOH, the equivalent was found to be 57–60 in contrast to 110–120 which was obtained by rapid solution of the compound in the alkali, followed by back titration. The lower values of the equivalent are probably due to the oxidation of the reductone released by hydrolysis.

A number of the condensation products with reductone have been found to be hemihydrated, monohydrated or dihydrated. The reductone sulphaguanidine condensation product is dihydrated and this is interesting since sulphaguanidine itself is monohydrated. Dehydro L ascorbic acid has recently been shown by Kenyon & Munro (1948) to be hydrated. This compound has a structure not unlike the keto form of the condensation products of reductone.

Reactions of reductone condensation products

The reductone *p* aminobenzoic acid condensation product reacts readily with phenylhydrazine (O'Meara *et al.* 1947) to give an osazone. This osazone has been prepared and analysed. It possesses a *p* aminobenzoic acid residue.

Woods (1940) showed that *p* aminobenzoic acid can inhibit the action of sulphonamide derivatives, its power of inhibition being greatest with sulphanilamide and least with sulphathiazole. In view of these results, we tried the effect of *p* aminobenzoic acid on the reductone sulphathiazole condensation product. When these compounds are mixed in hot aqueous sodium acetate which has a pH similar to that found under biological conditions, the sulphathiazole residue is quickly displaced and the reductone *p* aminobenzoic acid condensation product results, whereas the reverse process has not been found to take place under similar conditions. A corresponding result was obtained when the reductone sulphaguanidine condensation product was treated with *p* aminobenzoic acid. On the other hand, when the reductone *p* aminobenzoic acid condensation product is treated with maphenide (marfanil, *p* aminomethylbenzenesulphonamide) hydrochloride in the presence of excess sodium acetate the *p* aminobenzoic acid is rapidly replaced by maphenide, the reductone derivative of the latter being formed.

Reductone *p* aminobenzoic acid condensation product, *p*-2,3'-dihydroxyprop 2' enyldeneaminobenzoic acid. A solution of reductone (0.4 g) in water (5 ml.) was shaken with charcoal and filtered. It was then added, with shaking, to a filtered solution of *p* aminobenzoic acid (0.7 g) in a mixture of water (10 ml) and acetone (10 ml). The clear solution soon became dark red and orange needles were quickly deposited. After 3 hr the compound was collected, washed with acetone, water and finally acetone, until the filtrate no longer gave a reaction with *p*-dimethylaminobenzaldehyde. The product (0.45 g) crystallized from dilute acetic acid as orange yellow needles, *m* p 254° (Found C, 53.15, H, 5.2, N, 6.1. Calc for $C_{10}H_8O_4N$ H_2O C, 53.3, H, 4.9, N, 6.2%). It gave absorption maxima in ethanol at 284 $m\mu$ ($\log \epsilon$, 4.21) and 355.5 $m\mu$ ($\log \epsilon$, 2.74). On drying on the water bath, the orange-yellow needles became red with loss of 1 mol. of water.

Titration. The compound (0.057 g) in 24.9 ml 0.05N NaOH was allowed to stand for 15 min. at room temperature, when the solution had become colourless. It was then

titrated with 0.05N H_2SO_4 using phenolphthalein as indicator, when 14.3 ml of acid were required for neutralization. This gives an equivalent of 108 and mol wt of 216, assuming that the reductone is completely enolized. $C_{10}H_8O_4N \cdot H_2O$ requires mol wt 225 (compare the titration of reductone by Norrish & Griffiths, 1928), other estimations gave values of 240 and 228. When the compound was left in contact with 0.05N NaOH for 24 hr before titration, values of 62, 57 and 57 were found in three estimations.

Colorimetric estimation of purity of the reductone *p*-aminobenzoic acid condensation product. Ten ml of each of a series of aqueous solutions containing 3–15 mg/l of *p* aminobenzoic acid were added to 2 ml portions of a solution of Ehrlich reagent prepared by dissolving *p* dimethylaminobenzaldehyde (3 g) in a mixture of conc H_2SO_4 (7 ml.) and water (100 ml). The intensity of coloration in each case was measured by means of a Spekker absorptiometer using a blue filter. Concentration of *p* aminobenzoic acid was plotted against extinction on a graph. Reductone *p* aminobenzoic acid condensation product (15 mg) in 10 ml 0.1N NaOH was warmed for a few minutes and then diluted to 11–10 ml. of this solution were treated with 2 ml of the Ehrlich reagent and the extinction was again determined. The *p* aminobenzoic acid content determined from the calibration graph was 58.0%. $C_{10}H_8O_4N \cdot H_2O$ requires 60.9 and $C_{10}H_8O_4N$ 66.1%. Repeat estimations gave values varying from 57 to 58%.

Reductone-sulphathiazole condensation product 2-(*p* 2' 3' Dihydroxyprop 2' enyldeneaminobenzenesulphonamido)thiazole. Sulphathiazole (2.0 g) in acetone (30 ml.) containing a few drops of 80% acetic acid was added to reductone (0.5 g) in dilute acetone (30 ml.). After several hours the required compound was obtained as yellow needles, which, after washing with water, gave *m* p 232° (decomp) with some pre-softening (Found C, 43.0, H, 4.0. $C_{18}H_{11}O_4N_2S_2 \cdot \frac{1}{2}H_2O$ requires C, 43.1, H, 3.6%). This compound lost water when heated at 100° in a vacuum, after which the sulphathiazole residue was estimated by Ehrlich reagent (Found sulphathiazole, 80.0. $C_{18}H_{11}O_4N_2S_2$ requires sulphathiazole, 78.5%).

Reductone-sulphapyridine condensation product 2-(*p* 2' 3' Dihydroxyprop 2' enyldeneaminobenzenesulphonamido)pyridine. This compound was obtained as an orange amorphous powder which did not melt sharply but started to darken and decompose at 194° (Found C, 50.6, H, 4.5. $C_{14}H_{13}O_4N_3S_2 \cdot H_2O$ requires C, 49.9, H, 4.4%).

Reductone-sulphamezathine condensation product 4,6-Dimethyl 2-(*p* 2' 3' dihydroxyprop 2' enyldeneaminobenzenesulphonamido)pyrimidine. This compound was an amorphous green powder, *m* p 200–240° (decomp) (Found C, 48.2, H, 4.9, N, 14.8. $C_{18}H_{16}O_4N_4S_2 \cdot H_2O$ requires C, 49.2, H, 4.9, N, 15.3%).

Reductone sulphamethylthiazole condensation product 4-Methyl 2-(*p* 2' 3' dihydroxyprop 2' enyldeneaminobenzenesulphonamido)thiazole. This compound consisted of a yellow orange, amorphous powder which shrivels and decomposes at 166–168° (Found C, 45.2, H, 4.3. $C_{13}H_{12}O_4N_2S_2 \cdot \frac{1}{2}H_2O$ requires C, 44.8, H, 4.0%).

Reductone-sulphaguanidine condensation product *p*-(2' 3'-Dihydroxyprop 2' enyldeneaminobenzenesulphonyl)guanidine. This compound formed yellow needles, *m* p 152°, which crystallized from water (Found C, 37.5, H, 5.1. $C_{10}H_{12}O_4N_4S_2 \cdot 2H_2O$ requires C, 37.5, H, 5.0%).

Reductone-solusceptazine condensation product. Reductone *p*-(sodium- α -sulphoethyl)aminobenzenesulphonamide condensa-

tion product Reductone *p*-aminobenzenesulphonamide condensation product *p* (2' 3' Dihydroxyprop 2' enyldeneamino) benzenesulphonamide Reductone was found to displace the substituting groups in the N⁴ position in the first two compounds. As a consequence all three gave the same condensation product with reductone. The yellow condensation product was crystallized from hot water as orange radiating needles which darkened, shrank and finally decomposed at 170–172°. The compounds derived from each of the three sources did not depress the melting point of one another (In two analyses found C, 42.0, H, 4.4 C, 40.8, H, 4.6 C₁₆H₁₆O₄N₂S · H₂O requires C, 41.5, H, 4.6%).

Reductone diaminodiphenylsulphone condensation product Bis *p* (2' 3' dihydroxyprop 2' enyldeneamino)phenyl sulphone This compound consisted of yellow micropisms which started to decompose at 156°, but gave no definite m.p. (Found C, 50.7, H, 4.8 C₁₈H₁₆O₆N₂S · 2H₂O requires C, 50.9, H, 4.7%).

Osazone of the reductone p-aminobenzoic acid condensation product The solid obtained when a mixture of the condensation product (0.5 g), phenylhydrazine hydrochloride (1.0 g) and sodium acetate (1.0 g) in acetic acid was warmed on the water bath for 0.5 hr was collected and the osazone crystallized from ethanol as yellow needles, m.p. 188–189° (Found C, 68.3, H, 5.3, N, 17.0 C₂₂H₁₆O₅N₆ requires C, 68.6, H, 4.9, N, 18.1%). The *p*-aminobenzoic acid content was also estimated by hydrolysis with HCl. Phenylhydrazine was distilled in steam and the residue was treated with Ehrlich reagent and the depth of colour estimated as above (Found *p*-aminobenzoic acid, 36.0 C₂₂H₁₆O₅N₆ requires 35.6%).

Action of p-aminobenzoic acid on reductone sulphathiazole condensation product A mixture of the reductone sulphathiazole condensation product (0.5 g), *p*-aminobenzoic acid (0.5 g), sodium acetate (2 g) and water (5 ml) was boiled for a few minutes and filtered whilst hot. On cooling, a yellow precipitate separated and the solution exhibited the green fluorescence characteristic of the reductone *p*-aminobenzoic acid condensation product. The precipitate was thoroughly washed with hot water to remove *p*-aminobenzoic acid and sulphathiazole. On drying, the yellow solid became orange and gave m.p. 250°, undepressed by authentic reductone *p*-aminobenzoic acid condensation product.

Reductone maphenide (marfanil) condensation product *p* (2' 3' Dihydroxyprop 2'-enyldeneaminomethyl)benzenesulphonamide A mixture of reductone *p*-aminobenzoic acid condensation product, maphenide hydrochloride (0.5 g) sodium acetate (2 g) and water (5 ml) was boiled for 2–3 min. with stirring. The hot mixture was filtered and cooled, when a yellow green solid separated. It crystallized from ethanol as pale green needles, m.p. 183–184° (Found C, 46.9, H, 4.8 C₁₆H₁₂O₄N₂S requires C, 46.9, H, 4.7%). On hydrolysis of this compound and diazotization of the product, a solution was obtained which did not couple with β-naphthol. Reductone *p*-aminobenzoic acid condensation product, gives, by similar treatment, the expected azo compound.

DISCUSSION

The results here recorded provide chemical support for the theory advanced by O'Meara *et al.* (1944, 1947) that the sulphonamides act by uniting with reductone during the active phase of normal bacterial metabolism. According to the view of these authors,

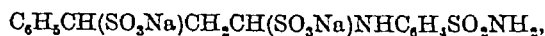
p-aminobenzoic acid is the normal cell metabolite which unites with reductone and stabilizes it prior to further utilization by the cell. If the place normally taken by *p*-aminobenzoic acid be occupied by a sulphonamide, cellular metabolism ceases.

We have found that under biological conditions of pH, *p*-aminobenzoic acid and all the sulphonamides tested readily form compounds with reductone. These compounds have been isolated and their constitution has been established. It is apparent that the aldehydic group of reductone undergoes condensation with the free primary aromatic amino group of *p*-aminobenzoic acid and the sulphonamides. It is interesting to note that reductone readily condenses with soluseptasine and *p* (sodium α-sulphoethyl)-aminobenzenesulphonamide to give, in each case, the reductone sulphanilamide condensation product. Northey (1940) has expressed the view that 'unless the substituting group in the N⁴ position is hydrolysed, reduced or otherwise removed *in vivo* it appears that the derivative will have little if any activity'. The chemical evidence here provided supports this view. Particularly interesting are the interactions of reductone sulphonamide condensation products with *p*-aminobenzoic acid and of the reductone *p*-aminobenzoic acid condensation product with maphenide (marfanil). Since, within the biological range of pH, *p*-aminobenzoic acid readily replaces the sulphonamides from their combinations with reductone, a chemical explanation is provided of the inhibition of the bacteriostatic action of the sulphonamides by *p*-aminobenzoic acid demonstrated by Woods (1940). Similarly, the replacement of *p*-aminobenzoic acid, from its combination with reductone, by maphenide, provides a chemical explanation of the facts that *p*-aminobenzoic acid does not antagonize maphenide (Schreuss, 1942; Goldacre, 1944) and that maphenide, unlike the sulphonamides, does not show a time lag in coming into operation (Jensen, Schmith & Brand, 1942). Moreover, these observations enable the mode of action of maphenide to be brought into line with that of the sulphonamides. Maphenide is a relatively strong base, and high concentrations are possible because of its high solubility in water. Both factors will operate so as to facilitate the replacement of *p*-aminobenzoic acid by maphenide in cellular metabolism.

We believe that the reductone *p*-aminobenzoic acid condensation product system is likely to prove very important in biological synthesis. Reference to biochemical processes, possibly involving reductone and the reductone *p*-aminobenzoic acid condensation product, has been made elsewhere (Bell, Cocker & O'Meara, 1948), and the synthesis of pteric acid and its 7-isomer by Forrest & Walker (1948) and of folic acid and its related compounds by Angier *et al.* (1948), lend colour to this view.

SUMMARY

1 Condensation products of reductone have been prepared with *p* aminobenzoic acid, 2 *p* aminobenzenesulphonamidothiazole (sulphathiazole), 2-*p* aminobenzenesulphonamidopyridine (sulphapyridine), 4,6 dimethyl 2 (*p* aminobenzenesulphonamido) pyrimidine (sulphamezathine), *p* aminomethylbenzenesulphonamide (maphenide, marfanil), 4 methyl 2 (*p* aminobenzenesulphonamido)thiazole (sulphamethylthiazole), *p* aminobenzenesulphonamylguanidine, 4,4' diaminodiphenylsulphone, *p* (disodium γ -phenyl α -disulphopropyl)aminobenzenesulphonamide (soluseptasine)



p-(sodium α sulphoethyl)aminobenzenesulphonamide ($\text{Me CH}(\text{SO}_3\text{Na})\text{NHC}_6\text{H}_4\text{SO}_2\text{NH}_2$) and *p* aminobenzenesulphonamide The condensation pro-

ducts of reductone with the three last compounds were identical

2 It has been shown that the condensation product of reductone and sulphathiazole is decomposed by *p* aminobenzoic acid The resultant product is identical with that obtained from reductone and *p*-aminobenzoic acid (*p* 2' 3' dihydroxy prop 2'-enylideneaminobenzoic acid), but the reverse process is apparently not possible under similar conditions Maphenide, however, displaces *p* aminobenzoic acid from *p* 2' 3' dihydroxyprop 2'-enylideneaminobenzoic acid

3 A theory of the mode of action of sulphonamide derivatives is discussed

One of us (E A B) was in receipt of a grant from the Sarah Purser Medical Research Fund

We desire to express our thanks to Imperial Chemical Industries Ltd and the Medical Research Council of Ireland for financial support

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The Micro-estimation of Citric Acid

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(Received 12 April 1949)

The estimation of citric acid by its conversion into pentabromoacetone (Stahre, 1895) has been greatly improved, notably by Pucher, Sherman & Vickery (1936), who introduced a sensitive colorimetric procedure. Recent modifications (Perlman, Lardy & Johnson, 1944; Krebs & Eggleston, 1944; Goldberg & Bernheim, 1944; Hunter & Leloir, 1945; Speck, Moulder & Evans, 1946; Taussky & Shorr, 1947,

Natelson, Lugovoy & Pincus, 1947, 1948; Wolcott & Boyer, 1948) have further increased the reliability and sensitivity of the method. However, the fact that modifications of the method continue to appear indicates that satisfaction is not general. Most authors agree that the addition of permanganate is a critical step which must be performed slowly and cautiously.

We became aware of the defects of the method in the course of an investigation of the effect of various mono-, di- and tri-carboxylic acids on the activity of aconitase. This involved the estimation of citric acid in the presence of a large excess of one or more other organic acids. Under these conditions the recovery of citric acid was apt to be low by up to 30%, a result which was but little affected by the various preliminary treatments recommended. It appears from the published data that, although interference with the specificity of the method has been thoroughly investigated, interference with recovery has received less attention.

In the modification here proposed, permanganate is replaced by vanadic acid as oxidizing agent. In our experience this modification has the advantage of greater simplicity, as the oxidizing agent may be added in bulk, of greater elasticity, as the amount of oxidizing agent need not be adapted to concentrations of citric acid varying within a wide range, and of greater freedom from interference by other substrates. In addition, the method is even more specific than the older one, for example, β hydroxybutyric acid gives no colour.

The use of vanadic acid was suggested by the observation of Pozzi Escot (1946), who found that the oxidation of citric acid to acetonedicarboxylic acid by chlorate was catalysed by a trace of vanadic acid. The function of chlorate in this reaction is probably that of reoxidizing the reduced vanadyl ion, and it might be expected that in the presence of a sufficient quantity of vanadate the oxidation would proceed in the absence of chlorate. This is indeed the case.

Reagents

METHOD

Sulphuric acid, 27N H_2SO_4 .

Bromine water, saturated.

Ammonium vanadate, 2% solution, dissolve by warming. The substance tends to crystallize on standing, and before use should be redissolved by warming.

Sodium thiosulphate, 5% solution.

Sodium sulphide (A.R.), 2% solution, freshly prepared.

Sodium sulphate (anhydrous).

Light petroleum, b.p. 80–100°. Purification: extract repeatedly with conc. H_2SO_4 , then with a saturated solution of KMnO_4 in 0.5N H_2SO_4 and finally with a 5% solution of FeSO_4 in 0.5N H_2SO_4 , wash with water, dry and rectify.

Citric acid standard, 1 mg of the crystalline acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$)/ml. (in N H_2SO_4).

Procedure

(a) *For quantities of 0.1–1.0 mg citric acid*. The solution (5 ml, containing not more than about 1 mg citric acid) is mixed with 27N H_2SO_4 (5 ml) in a 30 ml test tube. The mixture is cooled to room temperature, and bromine water (2 ml.) and ammonium vanadate solution (3 ml.) are added. The contents are well mixed and the stoppered tube is left in a water bath at 50° for 20 min. A slowly increasing turbidity indicates the formation of pentabromoacetone. The tube is again cooled to room temperature and excess Br_2 is removed

by adding 10–12 drops of $\text{Na}_2\text{S}_2\text{O}_3$ solution. The solution, which is now blue or bluish green, is extracted with 6 ml light petroleum by mechanical shaking of the stoppered tube for 2–3 min. The aqueous layer is blown out with the aid of a wash bottle head, i.e. a rubber stopper fitted with two glass tubes, of which the longer one should be smoothly adjustable so as to reach to the bottom of the test tube. This glass tube is tapered at both ends and the lower tip is ground smooth to ensure an even contact. During its introduction through the petroleum layer the delivery tip is closed with a finger. When most of the aqueous layer has been blown out the meniscus is allowed to rise slowly in the tube to the delivery tip. Sharp separation is thus achieved. After the addition of a pinch of anhydrous Na_2SO_4 the light petroleum extract is decanted into a clean test tube. Aqueous drops, if any should remain, must be prevented from running out. A 5 ml portion of the extract is now transferred to a third test tube and shaken for 1 min with 10 ml of Na_2S solution. The coloured aqueous layer is blown on to a funnel fitted with a dry filter paper, and the absorption of the clear filtrate is measured in the Spekker absorptiometer or a similar instrument, using 1 cm cells and Ilford no. 601 (spectrum violet) filters. Na_2S solution is used as a solvent blank. A calibration curve is determined in the usual way.

(b) *For quantities of 0.02–0.2 mg citric acid*. The simplest way of increasing the sensitivity of the method is to reduce the volume of the Na_2S extract. The extent to which this is possible depends mainly on the minimum volume required in the colorimetric instrument available. For quantities of 20–200 μg citric acid, with a photoelectric test-tube colorimeter (Hilger 'Biochem' absorptiometer), about 3 ml. are needed. The preparation of the pentabromoacetone extract is as described for the 0.1–1.0 mg range. A 5 ml sample of the extract is measured into a colorimeter tube and shaken with 3 ml Na_2S solution. The tube is centrifuged lightly for 1 min. Readings are taken without removing the petroleum layer.

Comments

Precautions. The use of test tubes permits the simultaneous analysis of a large number of samples. The use of a measured portion of the light petroleum extract makes it possible to dispense with repetition of extractions and with washing operations (cf. Natelson *et al.* 1947). Unsatisfactory results can usually be traced to one of two sources of error: incomplete removal of free Br_2 or contamination of the petroleum extract with acid. The former is recognized by the coloration of the petroleum extract, unless this is completely colourless; it is better to discard the sample than to complete the reduction of Br_2 by a further addition of thiosulphate. The use of thiosulphate is preferable to that of hydrazine sulphate, as its action is instantaneous and not accompanied by gas evolution which may be troublesome during the subsequent extraction. Contamination with acid may be due to faulty separation or to insufficient cleaning of wash bottle heads or stoppers. It is advisable to keep separate sets of these for the two extractions.

Colour stability. The addition of a stabilizing solvent to the Na_2S extract is unnecessary, likewise no advantage was found in using ice-cold Na_2S solutions, since lower readings were obtained than with solutions at room temperature. For all practical purposes the colour is stable for at least 30 min. at about 18°, especially if protected from strong light. Data on colour fading are contained in Table 1. The fading is slower in the more concentrated solutions.

Table 1 Colour fading of sodium sulphide extracts after shaking with light petroleum solutions of pentabromoacetone

Temperature (°)	Exposure to daylight	Time of measurement (hr)							
		0	1	2	3	4	5	21	46
		Extinction coefficients							
17-20	+	0.100	0.087	0.081	0.075	0.072	0.071	0.043	—
	—	0.100	0.102	0.099	0.093	0.093	0.086	0.050	—
	+	0.201	0.178	0.155	0.153	0.150	0.147	0.087	0.063
	—	0.201	0.201	0.191	0.188	0.186	0.171	0.103	0.077
	+	1.00	0.99	0.92	0.89	0.87	0.83	0.58	0.390
	—	1.00	1.00	0.96	0.94	0.92	0.90	0.65	0.460
3	—	0.965	0.980	—	—	—	—	—	0.585

The light sensitivity of the colour is a little troublesome when the absorption is measured in tubes with the Hilger 'Biochem' absorptiometer. Here the incident light beam is concentrated on the solution in a sharp focus by a condensing lens. This causes a slow, but completely reversible, decrease of light absorption. Reliable results can be obtained by taking quick readings and by twisting the tube slowly during exposure, thus continuously mixing irradiated and unirradiated parts of the solution. No such colour instability was noticed when flat-sided cuvettes were used.

Accuracy The calibration curve is a straight line going through the origin. The standard deviation of an estimation is well below 2% within the range of 0.2-1.0 mg citric acid (Table 2). Standardization of the temperature (18°) and duration (1 min.) of the Na_2S extraction is important for obtaining reproducible results. If the extraction is prolonged for more than 1 min., a progressive reduction of colour intensity occurs.

Table 2 Analyses of pure citric acid solutions

(Extraction by 10 ml Na_2S solution, Ilford filter no 601)

Cryst citric acid (mg)	No of estima- tions	Extinction coefficient (mean)	Standard deviation	Yield of penta bromo acetone (%)
0.10	10	0.1034	0.00414	103
0.20	10	0.2029	0.00314	101
0.30	10	0.3075	0.00425	102
0.50	10	0.5025	0.00403	100
1.00	10	0.9820	0.00350	100

The colour intensity corresponds to a yield of pure pentabromoacetone of slightly over 100%. This is probably connected with the fact that, besides pentabromoacetone, small quantities of other bromoacetones, notably hexabromoacetone, are formed (Goldberg & Bernheim, 1944).

Specificity The following compounds, in amounts of 0.1 mmol, gave no colour in the test: glucose, fructose, L-tyrosine, L-phenylalanine and the following acids: glyoxylic, L-lactic, pyruvic, malonic, succinic, fumaric, maleic, tartaric, DL-malic, L-tartaric, α ketoglutaric, L- α hydroxyglutaric, crotonic, (\pm) β hydroxybutyric, tricarballic, citraconic, *cis* and *trans* aconitic, (\pm) isocitric, DL-threo and DL-erythro 2,3-dihydroxybutyric, L-glutamic, L-aspartic and isoacetic.

Solutions of acetoacetic acid containing more than 0.01 mmol/ml give a yellow colour, but not after a preliminary boiling with acid. A specimen of oxaloacetic acid also gave a positive reaction, 1 ml of a 0.1 M solution simulating 0.225 mg citric acid. This reaction was unaffected by preliminary boiling with acid and it may have been due to contamination of the specimen with citric acid or a related substance. Itaconic acid, in contrast to citraconic acid, produces a yellow colour, 1 ml of a 0.1 M solution being equal in this respect to 0.80 mg citric acid. This value was not changed by repeated recrystallization, it is, however, much smaller than that reported by Breusch & Tulus (1947).

In the presence of certain aromatic compounds, especially salicylic acid, it is necessary to carry out a preliminary bromination and to remove the bromination product by filtration or by extraction with light petroleum.

Recovery The following substances, in amounts of 0.1 mmol, did not interfere with the recovery of 1 mg citric acid by the usual procedure: glucose, pyruvic, succinic, fumaric, maleic, *cis* and *trans* aconitic, glutamic and β hydroxybutyric acids. In the presence of some other substances, notably hydroxy acids, but also fructose, malonic and α ketoglutaric acids, recoveries were only quantitative if an excess of Br_2 and vanadate was maintained. Undue reduction of Br_2 is recognized by the colour of the solution veering towards green during incubation at 50°. In this case more Br_2 is added in the form of a saturated solution of Br_2 in 9N H_2SO_4 , in portions of 1-2 ml, until the orange colour of the solution, or brown vapours above it, persist. Additional Br_2 (1-7 ml. of the solution) was required in presence of 0.1 mmol of the following substances: fructose, malonic, lactic, malic, α hydroxyglutaric, α ketoglutaric, oxaloacetic, isocitric, tartaric, tartaric and 2,3 dihydroxybutyric acids. Provided the addition of bromine was early and sufficient, recoveries of citric acid were quantitative. In these experiments a 3% solution of ammonium vanadate was used.

The presence of any of the substances mentioned in high concentration is a complication which may require some adjustment of the standard procedure as far as the amounts of Br_2 and vanadate solutions are concerned. The vanadic acid method is more adaptable in this respect and gives better results than the permanganate method.

Pre-treatment with periodic acid Some interfering substances may be removed by a preliminary treatment with periodic acid. These include not only α glycols, such as tartaric acid, or polyhydroxy compounds, such as mono or di saccharides, etc., but also glyoxylic, malonic and tartaric.

acids (Sprinson & Chargaff, 1946) The following procedure gave quantitative recovery of citric acid (1 mg) in the presence of 0.1 mmol. of fructose or tartaric acid. 0.5 ml. of a saturated solution of sodium periodate is added to 3–5 ml. of the neutral solution to be analysed. After standing at room temperature for 15 min. the solution is acidified with 0.3 ml. 10N H_2SO_4 and a 2M solution of KI is added dropwise, until the solution is faintly yellow. Iodate formed in the reaction is thereby reduced to I_2 . After addition of 0.1 ml. 6% H_2O_2 (to reduce excess periodic acid to I_2) the solution is measured, filtered from separated I_2 and extracted twice with 3 ml. light petroleum. A sample of the filtrate (3–4 ml.) is mixed with 0.2 ml. 30% KBr (to remove excess H_2O_2), made up to 5 ml. and analysed by the usual procedure.

Modifications of the estimation of pentabromoacetone. Some recent modifications of citric acid analysis concern the estimation of pentabromoacetone. This substance is estimated either by its colour reaction with Na_2S or related compounds, or by procedures in which inorganic bromide is liberated and measured by titration or by optical methods. The latter procedures are less specific than the former, as the extract may contain other bromination products which liberate bromide, but do not give the colour reaction with sulphide (cf. Breusch & Tulus, 1947). In the method of Tausky & Shorr (1947) the solution of pentabromoacetone is shaken with ethanolic NaI solution and the liberated I_2 is measured optically. The colour intensity produced, and therefore the sensitivity of the method, is not superior to that of the Na_2S method. The method has further the great disadvantage that the colour intensity slowly increases for several hours (Table 3).

Table 3 *Extinctions of various extracts (10 ml.) after shaking with light petroleum solutions of pentabromoacetone (0.897 mg in 5 ml.)*

Extraction by	Ilford spectrum filter no	Time of measurement (hr)			
		0	1	2	3
		Extinction coefficients			
Na ₂ S (2%)	601	0.500	0.490	0.475	0.460
Ethanolic NaI (10%)	601	0.445	0.500	0.530	0.540
Thiourea (4%) in borax (2%)	601	0.575	0.420	0.380	0.365
Thiourea (4%) + Na ₂ S (0.06%)	603	0.540	0.540	0.530	0.533

Natelson *et al.* (1948) have advocated the use of thiourea at pH 9.2, instead of Na_2S . They claim that the colour is stable at room temperature, and that the extinction coefficient is approximately twice that of the Na_2S colour. Using the Spekker absorptiometer with Ilford filter no. 601 (maximum transmission about 440 $\text{m}\mu$) we obtained, with the method of Natelson *et al.*, an extinction coefficient only 10–15% higher than with Na_2S , but the stability of the colour was considerably less (Table 3).

Natelson *et al.* observed a different colour (absorption maximum at 510 $\text{m}\mu$) after prolonged shaking of a neutral thiourea solution with a light petroleum solution of pentabromoacetone. We found that this colour develops rapidly, in about 0.5 min., if the thiourea solution contains 0.06% Na_2S . Under these conditions fading was indeed reduced to a minimum. The extinction coefficient, read with Ilford filter no. 603 (maximum transmission at about 500 $\text{m}\mu$), was, however, substantially the same as that obtained by other methods (Table 3). According to Natelson *et al.* the pink colour (absorption maximum 510 $\text{m}\mu$) of neutral thiourea

solutions changes to the yellow colour (absorption maximum 460 $\text{m}\mu$) at alkaline pH and vice versa. However, with a 4% thiourea 2% borax solution containing 0.06% Na_2S the same pink colour is produced as with a neutral thiourea solution.

At present we are not sufficiently impressed by the advantages of the thiourea method to substitute it for the well tried sulphide method.

Estimation of citric acid in biological fluids

Serum or plasma. The sample (3 ml.) is added slowly, with shaking, to 10% trichloroacetic acid (25 ml.). A portion of the filtrate (20–22 ml.) is transferred to a small conical flask containing one or two glass beads and the neck is lightly plugged with glass wool to prevent splashing. The solution is boiled down to 2–3 ml. on a hot plate and the residue is poured into a test tube and made up to 5 ml. with the rinsings of the flask and glass wool. The further analysis is carried out according to the procedure for the 0.02–0.2 mg. range. Recovery of added citric acid was good (Table 4). A number of analyses on human sera gave results between 15 and 35 $\mu\text{g}/\text{ml}$.

Recently, microcells for use with the Spekker absorptiometer became available to us. They permit estimations on 0.5 ml. solution and proved very useful for the estimation of citric acid in serum. The light petroleum extract was shaken with 1 ml. 2%

sodium sulphide solution and about 0.5 ml. of the coloured aqueous layer was withdrawn with a test pipette and placed in the microcell. When using this

Table 4 *Recovery of citric acid from horse serum*

Citric acid (μg)			Recovery (%)
Added	Found	Recovered	
0	29.2	—	—
20	48.2	19	95
40	69.0	30.8	99.8
60	88.8	59.6	99.3
80	109.0	79.8	99.8

modification we had difficulties with trichloroacetic acid as deproteinizing agent. Metaphosphoric acid, too, was unsatisfactory, but tungstic acid filtrates gave consistent results. A disadvantage of tungstic acid filtrates is the appearance of a precipitate of

excess tungstic acid in the strongly acid solution in which the oxidation and bromination of citric acid is carried out. This does not interfere with the reaction, but it makes the removal of the aqueous layer by blowing out impossible. Instead, the petroleum layer is sucked off.

Cerebrospinal fluid The c.s.f. (1 vol.) is mixed with 30% trichloroacetic acid (0.1 vol.), and 1–3 ml of the filtrate are directly analysed without further pre-treatment. Range 40–80 $\mu\text{g/ml}$ (cf Benni, Larsson & Thunberg, 1943).

Urine Protein, if present, is removed by precipitation with trichloroacetic acid, turbidities by filtration, acetoacetic acid by boiling with dilute acid, and aromatic compounds, such as salicylates, by preliminary bromination. No preliminary treatment whatever is required for normal urines which

are analysed directly after suitable dilution. Samples of 1–5 ml of urine diluted fivefold usually give satisfactory readings. Recovery of added citric acid was quantitative.

SUMMARY

1 A modification of the colorimetric estimation of citric acid is described in which permanganate is replaced by vanadic acid. This gives increased simplicity and speed of operation, and greater freedom from interference by other oxidizable substrates. The yield of pentabromoacetone is almost quantitative.

2 Some recent modifications of the colorimetric estimation of pentabromoacetone are critically examined.

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Studies in Detoxication

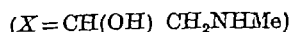
27 THE ORIENTATION OF CONJUGATION IN THE METABOLITES OF 4 CHLOROCATECHOL AND 4 CHLORORESORCINOL, WITH SOME OBSERVATIONS ON THE FATE OF (+) ADRENALINE, PROTOCATECHUIC ACID AND PROTOCATECHUIC ALDEHYDE IN THE RABBIT

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(Received 6 May 1949)

Garton & Williams (1948) have shown that catechol gives rise to monoconjugates, *o*-hydroxyphenylglucuronide and *o*-hydroxyphenylsulphuric acid, in the rabbit. Quinol and resorcinol also form monoconjugates in the rabbit (Garton & Williams, 1949). The object of the present work was to find out which of the two hydroxyls becomes conjugated if a third group *X* is present in a position *para* to one of the

hydroxyls in catechol compounds. We first studied protocatechuic aldehyde ($X = \text{CHO}$), protocatechuic acid ($X = \text{COOH}$) and (+) adrenaline



These three compounds formed glucuronides in the rabbit, but we did not succeed in isolating them in a crystalline state. The structure of these glucuronides could not therefore be rigidly proved. With 4-chlorocatechol ($X = \text{Cl}$), however, we succeeded in isolating in high yield a crystalline monoglucuronide whose structure could be determined. The structural

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problem amounted to proving that the isolated glucuronide was either 4 chloro- (I) or 5 chloro 2 hydroxyphenylglucuronide (II)



Since chlorocatechol formed a monoconjugate, it was interesting also to know whether 4 chlororesoreinol and chloroquinol formed monoconjugates, because the formation of monoconjugates in these compounds raises interesting questions concerning the orientation of conjugation. The study of the isolation and structure of 4 chlororesoreinol glucuronide is also included in this paper. In this case the structural problem involved consisted in proving that 4 chlororesoreinol glucuronide was either 4 chloro or 6 chloro-3 hydroxyphenylglucuronide. Our studies on chloroquinol will be reported in a future paper.

METHODS AND MATERIALS

Materials 4-Chlorocatechol hemihydrate (m p 81°) was prepared according to Willstätter & Müller (1911), and 4-chlororesoreinol (m p 105°) according to Reinhard (1878) (cf Moore, Day & Suter, 1934) (+) Adrenaline (m p 210°, $[\alpha]_D^{20} +50^\circ$ in 0.1 N HCl) was the gift of Burroughs Wellcome and Co. Protocatechuic aldehyde (m p 154°) was a commercial sample. Protocatechuic acid (m p 197–198°) was prepared in good yield by adding vanillin in small quantities to fused KOH, the method being a slight modification of that of Tiemann & Haarmann (1874).

Animals The rabbits used were kept on a diet of either 50 g or 75 g/day of Lever's cubes with water *ad lib*.

Analytical methods Etheral sulphates and glucuronic acid in urine were determined as in earlier papers in this series (Williams, 1938, Hanson, Mills & Williams, 1944).

Reference compounds 4-Chloro 2 methoxyphenyl benzoate (m p 78–79°) was prepared according to Jona & Pozzi (1911).

Synthesis of 4-chloro 3 methoxyphenol This compound was originally described by von Auwers & Pohl (1914–15), who obtained it in small yield by a lengthy procedure. We prepared it by a Sandmeyer reaction from 4-amino 3 methoxyphenol. Resoreinol monomethyl ether (Dey, 1935) was converted through *p* sulphophenylazo *m* methoxyphenol to 4-amino 3 methoxyphenol (Heidelberger & Jacobs, 1910). CuCl obtained from 12.5 g CuSO₄ · 5H₂O was dissolved in 20 ml conc HCl. This solution was added to the solution of the diazonium compound obtained by treating 3 g 4-amino 3 methoxyphenol dissolved in 30 ml 5–6 N HCl with 1.5 g NaNO₂ at –5°. The mixture was then refluxed for 45 min, whereby N₂ was liberated and a dark coloured oil separated. This oil was extracted with ether and the extract well washed with water. The phenol was now transferred to 2 N NaOH by extraction and finally the alkaline extract was acidified with 2 N HCl and the phenol extracted with ether. After drying with anhydrous Na₂SO₄, treating with charcoal and evaporating the ether, a dark coloured gum was obtained

which slowly crystallized (yield, 1.4 g). On distillation in a vacuum (140–150°/ <1 mm) 0.8 g of 4 chloro 3 methoxyphenol was obtained. After recrystallization from acetone water the phenol was obtained as white plates, m p 77–78° (found OMe, 19.6. Calc for C₇H₇O₂Cl OMe, 19.6%) Von Auwers & Pohl (1914–15) give m p 79–80°.

4-Chloro 3 methoxyphenyl *p* toluenesulphonate was obtained in good yield in the usual manner using aqueous NaOH and *p* toluenesulphonyl chloride. It formed plates, m p 65–66°, after recrystallization from aqueous ethanol (Found C, 53.9, H, 4.2, Cl, 11.0, S, 10.0. C₁₄H₁₃O₄SCl requires C, 53.8, H, 4.2, Cl, 11.3, S, 10.3%)

RESULTS

Table 1 shows that both 4 chlorocatechol and 4-chlororesoreinol were excreted completely conjugated with glucuronic and sulphuric acids, the ratio glucuronide/etheral sulphate being about 6 for chlorocatechol and 3 for chlororesoreinol. With protocatechuic aldehyde and acid, however, there was by no means a complete conjugation, for just over 40% of the aldehyde and about 30% of the acid could be accounted for by conjugation.

In the case of the aldehyde, large amounts of free protocatechuic acid could be isolated from the acidified urine by extraction with ether. This acid no doubt accounts for the rest of the aldehyde. It was also found that two glucuronides were being excreted, one which could be isolated as a gum and appeared to be a protocatechuic acid glucuronide and the other a protocatechuic aldehyde glucuronide which could be isolated as a 2,4-dinitrophenyl-hydrazone. As these glucuronides were difficult to purify, they were not further studied.

In the case of protocatechuic acid, a considerable amount of this acid can be extracted from the acidified urine with ether. In one experiment we recovered in crystalline form some 30% of the dose. It appears, therefore, that about one third of the acid fed appears in the urine conjugated and the rest in the free state.

The results with (+) adrenaline are different from all the others. The etheral sulphate figures are probably not significant, whereas the glucuronic acid figures suggest that some 20% of the adrenaline fed was excreted as glucuronides. We spent a considerable time studying the nature of these glucuronides, but we did not reach definite conclusions because the glucuronides were non crystalline. We were, however, able to show that two glucuronides were being excreted, one being a (+) adrenaline glucuronide giving (+) adrenaline on hydrolysis, and the other a nitrogen free glucuronide containing a catechol-like substance, as indicated by absorption spectrophotometry. The second glucuronide no doubt arises from deamination products of adrenaline and it can be separated chromatographically from the first glucuronide. Colour tests indicated that, in the

Table 1 *Glucuronic acid and ethereal sulphate excretion of rabbits receiving 4 substituted catechols orally*

Compound fed	Rabbit no	Wt (kg)	Dose (mg)	Ethereal sulphate (mg SO ₄ /day)		Glucuronic acid (mg/day)		Dose (%) excreted as	
				Average normal	'Extra'	Average normal	'Extra'	Ethereal sulphate	Glucuronic acid
4-Chlorocatechol*	143	2.9	430	35.8	31.2	247	493	14.0	91.0
	145	2.5	370	34.8	41.2	208	407	21.4	87.0
	146	2.15	320	30.0	18.0	217	353	10.5	84.5
4-Chlororesorcinol†	128	3.0	450	18.2	51.1	124	476	20.5	78.8
	129	3.0	450	19.5	71.4	151	459	28.6	76.0
	130	3.0	450	18.3	57.4	182	484	23.0	80.0
Protocatechuic aldehyde‡	83	3.1	775	18.8	90.7	121	273	20.2	25.1
	114	2.7	636	20.7	50.8	108	242	14.1	27.1
	117	2.4	601	20.2	51.3	123	239	14.7	28.1
Protocatechuic acid‡	80	3.1	774	25.3	62.1	—	—	15.5	—
	81	2.45	613	24.0	44.2	—	—	13.9	—
	124	3.3	825	29.3	40.1	80	160	9.4	15.4
	125	3.6	900	11.6	76.8	88	219	16.4	19.4
	126	3.6	900	17.3	53.1	102	220	11.4	19.4
(+) Adrenaline†	68	2.85	710	17.0	13.3	147	142	4.3	18.9
	102	2.9	730	22.6	21.5	148	153	6.7	19.7
	101	2.85	713	33.0	0.0	—	—	0.0	—
	60	2.55	510	25.5	0.0	160	103	0.0	19.1
	67	2.5	500	30.8	15.0	149	157	6.9	29.6
	68	2.9	580	—	—	174	126	—	20.5

* Diet 75 g Lever's cubes/day

† Diet 50 g Lever's cubes/day

‡ The results quoted for these compounds were obtained by Dr G. A. Garton

gummy (+) adrenaline glucuronide, the glucuronic acid molecule is attached to one of the phenolic hydroxyl groups of adrenaline

THE METABOLITES OF 4-CHLOROCATECHOL

The structure of 4-chlorocatechol glucuronide

The glucuronide gum Twelve rabbits received collectively 9 g of chlorocatechol hemihydrate (0.75 g each) dissolved in water. The 24-hr urine (1200 ml.) appeared normal in colour and gave an intense Tollens test for glucuronic acid but no reaction with FeCl₃ or Benedict reagent. It was made acid to Congo red with dilute H₂SO₄ and saturated with (NH₄)₂SO₄. To the mixture 50 ml of 1:5 ethanol ether were added, and the precipitated salts filtered off. The clear filtrate was now exhaustively extracted in a separating funnel with 200 ml. portions of the 1:5 ethanol ether mixture. After drying over anhydrous Na₂SO₄, the yellowish extract was treated with charcoal and filtered. The filtrate was taken to a dark gum (15 g) *in vacuo* at a low temperature. This gum, which is presumably 4-chlorocatechol glucuronide, did not crystallize. It formed a crystalline salt (m.p. 108–109°) with benzylamine which appeared to contain two molecules of benzylamine. This salt was, however, unstable. 4-Chlorocatechol itself formed a crystalline salt (m.p. 61–62°) with benzylamine. These salts were not further investigated owing to their instability.

The glucuronide gum gave an intense Tollens test but no colour with FeCl₃. With 2,6-dichloroquinonechloroimide it gave a deep blue colour at pH 7. The latter test indicates the presence in the compound of a free phenolic hydroxyl group with the position *para* to it unsubstituted.

4-Chloro 2-methoxyphenylglucuronamide The above gum (12 g) was dissolved in 100 ml absolute ethanol and repeatedly methylated with excess ethereal diazomethane for 12 hr periods at room temperature until the colour test with 2,6-dichloroquinonechloroimide was negative. Filtration and removal of the solvents left a clear gum which did not crystallize. The gum appears to be 4-chloro 2-methoxyphenyl glucuronide methyl ester (Found OMe, 19.4; C₁₄H₁₇O₇Cl requires OMe, 17.8%)

This ester (12 g) was dissolved in the minimum of absolute methanol and the solution saturated with dry NH₃ at 0°. The flask was stoppered and kept at 0° for 12 hr. After that time the whole had set to a gelatinous mass, which crystallized on adding a few drops of acetone and scratching. The crystals (9.2 g, m.p. 216°) were filtered and washed with 30% ethanol from which solvent it crystallized as long colourless narrow plates, m.p. 218° and $[\alpha]_D^{20} - 96.15^\circ$ ($c = 0.2$ in 50% aqueous acetone). The 4-chloro 2-methoxyphenyl-β-D-glucuronamide monohydrate was insoluble in cold water, ether, light petroleum and benzene, slightly soluble in ethanol, soluble in acetone water mixtures and readily soluble in hot ethanol, water and acetone (Found C, 45.0, H, 5.2, N, 3.9, Cl, 10.0, H₂O, 4.6; C₁₄H₁₆O₇NCl · H₂O requires C, 44.4, H, 5.2, N, 4.0, Cl, 10.1, H₂O, 5.1%). The yield of this amide corresponded to 60% of the chlorocatechol fed.

Hydrolysis of the above amide and isolation of 4-chloro guaiacol The amide (15 g) was heated on a boiling water bath for 5 hr with 50 ml N HCl. The amide gradually dissolved and eventually a brown oil separated. The mixture was cooled and extracted with ether. After keeping overnight over anhydrous Na₂SO₄, the ethereal extract was treated with charcoal and filtered. The now pale yellow filtrate was evaporated at room temperature to a partly crystalline solid

consisting of chloroguaiacol This was benzoylated with benzoyl chloride and NaOH On pouring the benzoylated mixture into water a solid (0.9 g) separated This was recrystallized (charcoal) from ethanol, yielding 0.65 g of colourless plates, m p 72° Further recrystallization from ethanol raised the m p to 78–79° and the compound was identified as 4-chloro 2-methoxyphenyl benzoate (Found OMe, 11.9 Calc for $C_{14}H_{11}O_3Cl$ OMe, 11.8%) It did not depress the m p of authentic 4-chloro 2-methoxyphenyl benzoate (m p 78–79°) prepared according to Jona & Pozzi (1911) The other isomer, 5-chloro 2-methoxyphenyl benzoate, melts at 56–58° (Jona & Pozzi, 1911)

The ethereal sulphate of chlorocatechol

The urine of ten rabbits which had received collectively 10 g of chlorocatechol was collected for 2 days (2.5 l) and then concentrated to 500 ml under reduced pressure The concentrate was saturated with $(NH_4)_2SO_4$ and extracted with 4×300 ml acetone These extracts, after the addition of 2 g K_2CO_3 to maintain the solution alkaline, were reduced to 200 ml *in vacuo* At this stage the material contained ethereal sulphate and some glucuronide, but gave no test with $FeCl_3$ Much of the glucuronide was thrown out as a gum on pouring the extract into 1 l dry acetone The acetone solution was now reduced to 100 ml and again poured into 1 l dry acetone In this way the whole of the glucuronide was removed and the final acetone solution was now reduced to 100 ml This solution was now diluted with 300 ml absolute ethanol and then treated with an ethanolic solution of anhydrous oxalic acid The precipitated urea oxalate was removed, the filtrate made alkaline with ethanolic KOH and then reduced to small bulk, and the whole process repeated In this way the urea present was removed Excess oxalic acid was removed by making the concentrate alkaline with ethanolic KOH and filtering off the precipitated potassium oxalate Finally, a dark brown syrup was obtained which reacted strongly for ethereal sulphates, but gave negative tests for glucuronide and inorganic sulphate The $FeCl_3$ test was negative, but became intensely positive for catechol on acid hydrolysis With 2,6-dichloroquinone chloroimide the gum gave an intense blue colour which suggested that the ethereal sulphate was 4-chloro 2-hydroxyphenylsulphuric acid We were unable to induce the gum to crystallize

The gum was therefore methylated in the usual way with methyl sulphate and NaOH until the blue colour given by 2,6-dichloroquinonechloroimide had disappeared, and in this way 1.5 g of a methylated gum containing ethereal sulphate was obtained The gum was hydrolysed by boiling for 20 min. with $3N$ H_2SO_4 and the phenol set free was extracted with ether The ether yielded 0.8 g of a reddish gum (Found OMe, 16.0 Calc for $C_7H_7O_2Cl$ OMe, 19.6%), which is presumably crude chloromethoxyphenol This material was benzoylated, but the product would not crystallize The benzoylated gum was therefore distilled at 80° in a vacuum (<1 mm) and the straw coloured distillate crystallized on seeding with authentic 2-methoxy 4-chlorophenyl benzoate On recrystallization from ethanol 14 mg of 2-methoxy-4-chlorophenyl benzoate m p and mixed m p 79° were obtained (Found OMe, 11.6 Calc for $C_{14}H_{11}O_3Cl$ OMe, 11.8%) This experiment suggests that the ethereal sulphate in chlorocatechol urine is 4-chloro 2-hydroxyphenylsulphuric acid

THE METABOLITES OF 4-CHLORORESORCINOL

4-chlororesorcinol glucuronide

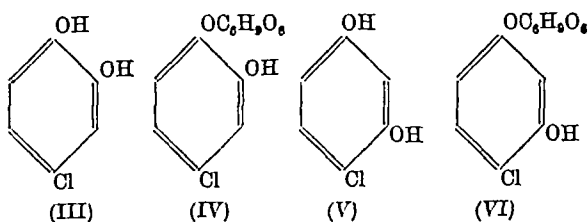
The glucuronide gum A total of 5.4 g of 4-chlororesorcinol was fed to six rabbits The urine (850 ml) was non-reducing, gave no colour with $FeCl_3$, but gave a strong naphthoresorcinol test The glucuronide gum (0.9 g), presumably 4-chlororesorcinol glucuronide, was obtained by ether ethanol extraction as described for chlorocatechol glucuronide

4-Chloro 3-methoxyphenylglucuronamide Repeated methylation of the gum with ethereal diazomethane yielded the non-crystalline ester, 4-chloro 3-methoxyphenylglucuronic acid methyl ester (Found OMe, 17.0 $C_{14}H_{17}O_6Cl$ requires OMe, 17.8%) Treatment of the ester in dry methanol with gaseous NH_3 yielded 6.2 g (equivalent to 50% of the dose) of crude 4-chloro 3-methoxyphenyl β -D-glucuronamide monohydrate The amide formed small plates from water with m p 214–215° and $[\alpha]_D^{19} = -103.3^\circ$ ($c = 0.2$ in 50% aqueous acetone) (Found C, 44.7, H, 5.3, N, 4.1, OMe, 9.0, H_2O , 4.7 $C_{13}H_{13}O_6NCl \cdot H_2O$ requires C, 44.4, H, 5.2, N, 4.0, OMe, 8.8, H_2O , 5.1%)

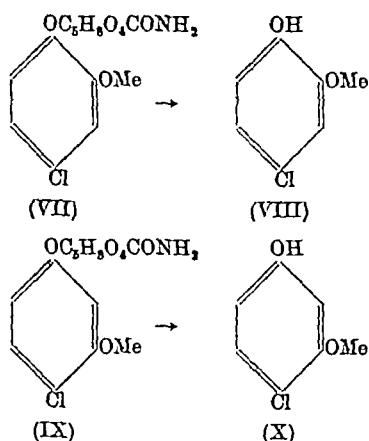
Hydrolysis of the above amide and isolation of 4-chloro 3-methoxyphenol The above glucuronamide (1.4 g) was hydrolysed by heating on the water bath for 6 hr with 50 ml 2N HCl The hydrolysate was black in colour and a black gum separated It appears that the hydrolysis is complicated by the combination of glucuronic acid and/or its degradation products with the phenol set free, as was found in the case of resorcinol glucuronide (Garton & Williams, 1949) The whole of the hydrolysate was exhaustively extracted with ether The extracts were washed with water and extracted with 2N NaOH The alkaline extract was now acidified with 2N HCl and the phenol extracted with ether After drying with anhydrous Na_2SO_4 and treating with charcoal, the ether was evaporated leaving 0.55 g of a black gum This gum was now subjected to vacuum distillation (<1 mm) The fraction distilling at 150–160° (bath temperature) was a pale straw coloured viscous oil (0.18 g) which crystallized On recrystallization from acetone water, the material was identified as 4-chloro 3-methoxyphenol, m p and mixed m p 77–78° (Found OMe, 19.3 Calc for $C_7H_7O_2Cl$ OMe, 19.6%) The *p*-toluenesulphonate was prepared and on recrystallization from aqueous ethanol yielded plates m p and mixed m p 65–66° with the authentic sample prepared above

DISCUSSION

It is clear that the glucuronides excreted by rabbits receiving 4-chlorocatechol (III) and 4-chlororesorcinol (V) are monoglucuronides, and can be described as 4-chloro 2-hydroxyphenylglucuronide (IV) and 4-chloro 3-hydroxyphenylglucuronide (VI), respectively



Neither of these compounds was obtained in a crystalline state, but they were readily characterized as the crystalline amides, 4-chloro-2-methoxyphenyl β -D-glucuronamide (VII, monohydrate, m.p. 218°) and 4-chloro-3-methoxy- β -D-glucuronamide (IX, monohydrate, m.p. 214–215°) which were obtained in excellent yields. The position of conjugation was determined by the hydrolysis of these amides and identification of the resulting chloromethoxyphenols. The amide (VII) yielded 4-chloro-2-methoxyphenol (VIII) characterized as the benzoate, and (IX) yielded 4-chloro-3-methoxyphenol (X) characterized as the *p*-toluenesulphonate.



The present results raise a number of interesting questions concerning the orientation of glucuronic acid conjugation. It has already been shown that in the rabbit catechol, resorcinol and quinol are conjugated on one hydroxyl only (Garton & Williams, 1948, 1949). This has now been shown to apply in the cases of 4-chlorocatechol and 4-chlororesorcinol, but with these compounds a further point arises because the two hydroxyls, in each case, are differently orientated in relation to the third substituent, chlorine. In both cases conjugation appears on the hydroxyl which is *para* to the chloro group. In 4-chlorocatechol the hydroxyl group *meta* to the chloro group is unconjugated, whereas in 4-chlororesorcinol the un-

conjugated hydroxyl group is *ortho* to the chloro group. It is thus clear that conjugation takes place at the hydroxyl which is farthest from the chloro group. Before, however, we can make definite statements about this orientation we must await results with chloroquinol in which one hydroxyl is *ortho* and the other *meta* to the chloro group. If the orientation of conjugation is simply a matter of distance then it can be predicted that chloroquinol will be conjugated at the *meta* hydroxyl.

It is interesting to compare the conjugation of chlorocatechol and chlororesorcinol with that of catechol and resorcinol. The relevant figures are given in Table 2 which shows that the total conjugation of the chloro compounds is higher than the dihydroxybenzenes. This is clearly the case with the resorcinols, although the ratio glucuronide/etheral sulphate for chlororesorcinol is approximately the same as for resorcinol. In the case of resorcinol, Garton & Williams (1949) found that at least 10% of the resorcinol fed could be recovered in the urine in the free state. With the two catechols, there appears to be a slight suppression of sulphate conjugation and a slight increase in glucuronic acid conjugation, on going from catechol to 4-chlorocatechol. However, in view of the variations in the individual results it would be safer to conclude that the introduction of the chloro group into the 4-position of catechol has but little influence on the conjugation of catechol, whereas the introduction of the chloro group into the 4-position of resorcinol increases both the etheral sulphate and the glucuronic acid conjugations.

SUMMARY

1 A study has been made of the conjugation of 4-chlorocatechol and 4-chlororesorcinol in the rabbit.

2 About 87% of 4-chlorocatechol is excreted as a glucuronide, which was characterized as the crystalline 4-chloro-2-methoxyphenylglucuronamide and proved to be 4-chloro-2-hydroxyphenylglucuronide. Some 15% of chlorocatechol forms a mono etheral

Table 2 The conjugation of catechol and resorcinol and their 4-chloro derivatives in the rabbit

Compound	Dose (mg/kg body wt.)	Dose (%) excreted as		G/E	Total conjugation (G + E)
		Etheral sulphate (E)	Glucuronide (G)		
Catechol*	100†	18	70	3.9	88
4-Chlorocatechol	150†	16.3	87	5.7	103
Resorcinol‡	100†	13.5	52	3.9	65
4-Chlororesorcinol	150†	24	78	3.3	102

* Figures quoted from Garton & Williams (1948)

† In approximately molecular proportions

‡ Figures quoted from Garton & Williams (1949)

sulphate which is 4 chloro 2 hydroxyphenylsulphuric acid

3 Similarly, 4 chlororesoreinol forms 78% of 4 chloro 3 hydroxyphenylglucuronide, characterized as 4 chloro 3 methoxyphenylglucuronamide. Some 24% is excreted as ethereal sulphate

4 The orientation of conjugation in these two compounds is discussed, and it appears that conjugation takes place at the hydroxyl group *para* to the chloro group

5 (+) Adrenaline forms 20% of glucuronide but

practically no ethereal sulphate. The results suggest that (+) adrenaline is conjugated on one of its phenolic hydroxyl groups

6 With protocatechuic acid, some 30% is conjugated and the rest is excreted unchanged

7 Protocatechuic aldehyde forms two glucuronides, is more highly conjugated than the acid and is largely transferred to protocatechuic acid and its conjugates

The expenses of this work were in part defrayed by a grant from the Medical Research Council

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Occurrence of Cytochrome and Coproporphyrin in Mycobacteria

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(Received 6 May 1949)

Yaoi & Tamiya (1928) and Fujita & Kodama (1934) observed a four banded spectrum in tubercle bacilli, the bands corresponding closely to those of reduced cytochrome in yeast or heart muscle. Frei, Riedmüller & Almasy (1934) confirmed the description in the case of *Mycobacterium tuberculosis hominis*, but observed a three banded spectrum lacking component *c* in strains of *Mycobact. avium* and *Mycobact. tuberculosis bovis*. In this laboratory it has been noticed that the absorption bands visible in suspensions of acid fast saprophytes vary in different species, some of which exhibit a distinct narrow band at 624 m μ . This band could be attributed to the presence of cytochrome *a*₂, alternatively, it could be due to free porphyrin, the other bands of which are obscured by the cytochrome spectrum.

Fischer & Fink (1925) extracted from heat killed tubercle bacilli a pigment that was identified spectroscopically as a metal complex of coproporphyrin (bands centred at 522.6 and 560.4 m μ). The spectrum of free coproporphyrin was not visible. The same authors, working with extracts of crude tuberculin, observed intense absorption bands attributed to protoporphyrin but 'only a very weak coproporphyrin spectrum'. Since the culture media were not

known to be free from porphyrin initially, Fischer & Fink (1925) did not regard their results as conclusive evidence of porphyrin formation by tubercle bacilli. The occurrence of porphyrin in acid-fast bacteria was demonstrated unequivocally by Dhéré, Glucksmann & Rapetti (1933), who observed the fluorescence spectrum of coproporphyrin in cells of *Mycobact. smegmatis*, *Mycobact. ranae*, *Mycobact. tuberculosis hominis* and *Mycobact. tuberculosis bovis* (strain B C G).

The work described in this paper, originally undertaken with the object of elucidating the atypical spectra of certain mycobacteria, led to the isolation of coproporphyrin III.

METHODS

Organisms The following were used: *Mycobact. smegmatis* (no. 523), *Mycobact. phlei* (no. 525), *Mycobact. sp. Karlinski* (no. 2071), *Mycobact. ranae* (no. 2801) and *Mycobact. stercoreis* (no. 3820). The numbers quoted are the catalogue numbers of the National Collection of Type Cultures, Lister Institute, London. The organisms were cultivated at 38° on meat infusion broth containing 2% (w/v) peptone and 5% (v/v) glycerol. Cultures were harvested on the seventh or eighth day, and washed thrice with distilled water (by centrifuga-

Table 1 *Absorption bands observable in suspensions of mycobacteria*

(Thickness of suspension, 1 cm Temp, 15–18° Presence or absence of each band is denoted by + or – respectively)

Organism	Band no Centre of band (m μ)	Absorption bands					
		1 624	2 598	3 570	4 564	5 552	6 526
<i>Mycobact phlei</i> (glycerinated broth)		–	+	–	–	–	–
<i>Mycobact phlei</i> (non glycerinated broth)		–	+	–	+	+	+
<i>Mycobact ranae</i>		–	+	–	+	+	+
<i>Mycobact Karlinski</i>		+	+	+	+	+	+
<i>Mycobact smegmatis</i>		+	+	+	+	+	+
<i>Mycobact stercois</i>		+	+	+	+	+	+

tion) before use in experiments which required intact organisms. Cultures used for extraction of porphyrin were harvested at the same age by decanting the broth. Bulk cultures were then washed thoroughly with several changes of water, transferred to a linen bag and pressed into a cake.

Spectroscopic examination of bacteria. The moist bacterial mass obtained by centrifuging was packed into an optical cell (internal width, 1 cm) and illuminated by a 250 W Philips projection lamp adequately housed. Protection against heat was afforded by a hollow glass cube (side, 3 cm) filled with water and put between the light source and the optical cell. A small condenser lens was used to concentrate light on the cell. The spectra were observed with a direct vision spectro scope (Schmidt & Haensch) of small dispersion equipped with a wavelength scale. The density of the suspension was adjusted by dilution with water until the definition of the bands was optimal.

Spectrophotometric measurements. Porphyrin solutions were examined in a Beckman quartz spectrophotometer, DU model. The absorption curve of coproporphyrin tetra methyl ester was determined in CHCl₃ solution. The ester was hydrolysed by standing in conc HCl and the absorption curve of the free porphyrin determined in 0.15N HCl (see Jope & O'Brien, 1945). The extinction coefficients of the free porphyrin ($E_{1\%}^{1\text{cm}}$) were measured at 401 and at 548 m μ , the respective slit widths being 0.04 mm (≈ 0.5 m μ at $\lambda = 400$ m μ) and 0.03 mm (≈ 0.9 m μ at $\lambda = 550$ m μ).

RESULTS

Absorption spectrum of acid fast saprophytes

The absorption bands seen in thick suspensions of five saprophytic mycobacteria are listed in Table 1. *Mycobact Karlinski* and *Mycobact stercois* consistently exhibit a sharply defined narrow band (1) at 624 m μ . This band is not present in all cultures of *Mycobact smegmatis*, and it is absent from the spectra of *Mycobact phlei* and *Mycobact ranae*.

Band 2, which is regularly present, extends from 592 to 603 m μ . Stretching from 551 to 568 m μ there is a zone of absorption which contains two dense bands centred at 552 m μ (5) and at 564 m μ (4) respectively. A fainter band (3), centred approximately at 570 m μ , is seen only in *Mycobact Karlinski*

and *Mycobact stercois*. Band 6, situated at 520–535 m μ , is partially obscured by end absorption. Bands 2, 4 and 5 were sharply defined in preparations which had been stored at –40°.

When *Mycobact phlei* is cultivated in glycerol broth it is so deeply pigmented with lipochromes that only one absorption band (2) can be identified, but when the organism is grown on plain broth it is possible to define bands 2, 4, 5 and 6. These results indicate that bands 2, 4 and 5 occupy the positions of the α bands of cytochromes *a*, *b* and *c*, while band 6 probably includes the β bands of the *b* and *c* components (Keilm & Hartree, 1939). The intensity of the blue end absorption made it difficult to search for the γ bands of the cytochromes.

Behaviour of the absorption bands

Effect of aeration. When oxygen was bubbled through suspensions of *Mycobact Karlinski* and *Mycobact stercois*, bands 2, 4, 5 and 6 disappeared, only to reappear after a few minutes of anaerobiosis. Bands 1 and 3 were unaffected, which suggests that they do not belong to a cytochrome complex.

The same phenomena were observed with *Mycobact smegmatis*, but prolonged aeration was necessary in order to oxidize the cytochromes. Filtered, moist air was passed for 18 hr through a sterile suspension kept at 37° in a thermostat. Spontaneous reduction at room temperature was slow, but the reduction time was diminished significantly by addition of lactate or malate (Table 2).

Table 2 *Reduction of cytochrome (Mycobacterium smegmatis)*

(Sterile suspension previously aerated for 18 hr. Reduction time assessed by visual observation of intensity of absorption bands.)

Substrate (neutral Na salt, 0.1M)	Reduction time at 18° (min.)
None	30–40
D,L-Lactate	14
L-Malate	15

Oxidation and reduction The cytochrome bands (2, 4, 5 and 6) disappeared on addition of potassium ferricyanide (two drops of saturated solution/2 ml bacterial suspension) in all species and reappeared after treatment with a small quantity of solid sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$). Bands 1 and 3 (where present) were unaffected by these reagents, nor did these bands change after addition of ammonium sulphide in the absence of ferricyanide (see Fujita & Kodama, 1934).

Effect of oxygen in presence of cyanide Bands 1 and 3 of *Mycobact Karlinski* and *Mycobact smegmatis* were unchanged when oxygen was bubbled through suspensions containing 0.002M hydrogen cyanide. Bands 2, 4, 5 and 6 persisted in the presence of oxygen and cyanide, behaving like typical cytochrome bands. The experiment shows that band 1 at 624 m μ is not due to cytochrome a_2 (see Negelein & Gerischer, 1934; Keilin, 1934; Fujita & Kodama, 1934).

Preparation of pyridine haemochromogen In suspensions of *Mycobact ranae*, *Mycobact Karlinski*, *Mycobact smegmatis* and *Mycobact stercoris* the cytochrome spectrum was replaced by that of pyridine haemochromogen (dense band at 548–562 m μ and weaker one at 525 m μ) following treatment with 2N potassium hydroxide, a few drops of 10% (w/v) aqueous pyridine solution and solid $\text{Na}_2\text{S}_2\text{O}_4$ added successively. Bands 1 and 3 were unaltered.

Extraction with acetone When a heavy suspension of *Mycobact Karlinski* was extracted with acetone, bands 1 and 3 disappeared from the spectrum; although bands 2 and 4 remained visible in moistened acetone powder. After dilution with water the acetone extract was acidified with acetic acid and shaken with ether. The ether layer was faint purplish red, gave a brilliant red fluorescence under a Wood lamp and showed a porphyrin spectrum with bands at 624, 570, 528 and 500 m μ .

These experiments show that the anomalous features of the absorption spectrum encountered in some species of mycobacteria are due to superposition of a porphyrin spectrum upon that of a cytochrome complex containing a , b and c components. Attempts to resolve band 2 into a and a_2 components by the use of carbon monoxide (Keilin & Hartree, 1939) were inconclusive.

Isolation of coproporphyrin from Mycobacterium Karlinski

Extraction Since the band at 624 m μ was most intense in *Mycobact Karlinski*, this organism was chosen for an attempt to isolate the porphyrin. Each batch of washed organisms, pressed into a cake weighing about 500 g, was stirred into 500 ml of cold acetone for 15 min and filtered on a Buchner

funnel. Extraction and filtration were repeated, once with 500 ml and twice with 250 ml of acetone, and the several filtrates combined.*

The acetone extract was diluted with 2 volumes of water, acidified with glacial acetic acid (3% of the total volume) and extracted four times with ether. The combined ethereal extracts were shaken with 1.4N hydrochloric acid, the ether separated, and the porphyrin stored in the acid solution until sufficient had been collected. Prior to storage residual ether was removed *in vacuo* at room temperature. The original acetone extract contained substantial amounts of lipids, which, being ether soluble, were largely removed during the transference of the porphyrin to the acid.

Preliminary purification In order to remove persistent traces of colourless lipid, the hydrochloric acid solution, representing extraction of 4.8 kg of bacterial press cake, was neutralized to congo red with solid sodium acetate, acidified with 0.05 volume of glacial acetic acid and the porphyrin transferred to ether. Transference between ether and hydrochloric acid was repeated four times in the usual way until the whole of the porphyrin was concentrated into 150 ml of 1.4N hydrochloric acid. Again residual ether was removed *in vacuo* at room temperature.

Sodium hydroxide (30%) was added slowly to bring the solution to pH 3.3 when the porphyrin flocculated. After standing overnight it was separated by centrifugation, dried *in vacuo* over concentrated sulphuric acid, dissolved in 15 ml of methanol previously saturated with dry hydrogen chloride gas and kept in the ice box for 24 hr. The solution of methyl ester was poured into 150 ml of chilled water, and the solution extracted repeatedly with small volumes of chloroform. The chloroform solution was washed twice with water, once with 2N ammonium hydroxide, and then three times with water. After filtration through a paper moistened with chloroform, the solution was evaporated to dryness *in vacuo*.

Chromatographic purification The crude ester, dissolved in a mixture of benzene (15 ml) and light petroleum (5 ml), was adsorbed on a calcium carbonate column (15 × 15 cm) according to the method of Grinstein, Schwartz & Watson (1945). A broad purple band of coproporphyrin methyl

* The completeness of acetone extraction was demonstrated by subsequent treatment of the dry powder from which acetone had been removed *in vacuo*. A sample was extracted with acetic acid and ether, and the ether shaken with a small volume of 2% (w/v) hydrochloric acid. Porphyrin could not be detected in the acid layer either spectroscopically or by fluorescence in ultraviolet light. The success of acetone extraction may depend on the solubility of porphyrin in acetone soluble lipids of the bacteria.

ester, which migrated slowly down the column, was eluted with the original solvent mixture. The ester was evaporated to dryness *in vacuo* at room temperature, crystallized from anhydrous ether and the crystals dried thoroughly *in vacuo* over concentrated sulphuric acid and paraffin wax.

Since a small amount of pigment remained on the upper and middle zones of the calcium carbonate, the column was cut and both zones extracted with chloroform. The upper zone yielded a quantity of coproporphyrin too small to permit isolation. The middle zone released a brownish red pigment (single absorption band at $645\text{ m}\mu$) which may be identical with a decomposition product of coproporphyrin described by Rimington (1939). The crystalline coproporphyrin ester was taken up in benzene and chromatography repeated by the method of Gray & Holt (1948). The eluate was evaporated to dryness *in vacuo* at room temperature, and the coproporphyrin ester twice recrystallized from anhydrous ether as before. Yield, 16 mg.

Properties of the crystalline ester. Dark red crystals, assuming the rosette pattern of coproporphyrin III tetramethyl ester (Rimington, 1939). Sharp m.p. at 150° , after cooling, remelt at $172\text{--}174^\circ$. HCl number, 1.5. Absorption curve (chloroform solution) in the visible region conformed to a 'step ladder' type with the chief band maxima at (I) 623.5 , (II) 567 , (III) 534 and (IV) $498.5\text{ m}\mu$. Relative intensities, $\text{IV} > \text{III} > \text{II} > \text{I}$.

Properties of the free porphyrin. Solutions in hydrochloric acid and in ether had the characteristic violet colour of coproporphyrin and gave a strong red fluorescence in ultraviolet light. HCl number, 0.1. Absorption curve in 0.15N -hydrochloric acid showed three bands with maxima at (I) 591 , (II) 548 and (III) $401\text{ m}\mu$. Relative intensities, $\text{III} > \text{II} > \text{I}$. In 0.15N hydrochloric acid, $E_{1\%}^{1\text{cm}}$ = 7400 at $401\text{ m}\mu$ and 243 at $548\text{ m}\mu$.

These data show that the crystalline material is a fairly pure specimen of coproporphyrin III tetramethyl ester. The extinction coefficients are about 9% lower than the maximum values quoted by Jope & O'Brien (1945) for recrystallized specimens of coproporphyrin methyl ester, both natural and synthetic. The melting points are within the range recorded for natural specimens.

Coproporphyrin content of mycobacteria

The coproporphyrin content of *Mycobact. Karlini* was determined by extracting 22 g of bacterial cake with acetone, transferring the pigment quantitatively through ether to 0.15N -hydrochloric acid and measuring $\log I_0/I$ at $548\text{ m}\mu$. Two transfers between ether and 5% (w/v) hydrochloric acid preceded the final concentration in 0.15N hydrochloric acid. The organism contained 5–8 mg of coproporphyrin/kg of bacteria, depending on the batch.

Protoporphyrin was not encountered at any stage during the isolation. If metal complexes or uroporphyrin were present, these, too, escaped detection.

In the course of this work it was found that the acetone powder contains an unstable haem pigment (possibly derived from cytochrome) which is incompletely removed by acetone hydrochloric acid. This pigment has a single absorption band at $545\text{--}555\text{ m}\mu$ and is readily converted into a pyridine haemochromogen.

Other species. The methods employed failed to extract coproporphyrin from *Mycobact. phlei* and *Mycobact. ranae*. Small quantities were obtained from *Mycobact. smegmatis*, *Mycobact. stercoris* contains considerably more than *Mycobact. smegmatis*, though less than *Mycobact. Karlini*. These findings were consistent with spectroscopic observations on the intact organisms.

Origin of coproporphyrin

Each 400 ml nutrient broth was extracted with acetic acid-ether and hydrochloric acid before inoculation to ensure that it was free from coproporphyrin. Spectroscopic and fluorescence tests did not reveal the presence of porphyrin. Tests on the broth for haematin by the conversion to pyridine haemochromogen were also negative.

The ability of *Mycobact. Karlini* to synthesize coproporphyrin was demonstrated by cultivating the organism in a synthetic medium composed of a basal salt mixture (Edson & Hunter, 1943) to which were added lactate (1%), glycerol (0.5%) and sodium citrate (0.001M , final concentrations). The porphyrin bands at 624 and $570\text{ m}\mu$, accompanied by those of the cytochrome spectrum, were seen in the bacteria, and acetone extracted the usual quantity of coproporphyrin.

DISCUSSION

Since the mycobacteria are strictly aerobic, exhibition of the four absorption bands of a typical cytochrome is to be expected. Indeed, Fujita & Kodama (1934) have classified the tubercle organism, along with *Bacillus subtilis* and other aerobic bacteria which possess *a*, *b*, *c* and *d* bands. The observations described in this paper show that *Mycobact. phlei* and *Mycobact. ranae* conform to group I of Fujita & Kodama's classification, whereas *Mycobact. Karlini*, *Mycobact. smegmatis* and *Mycobact. stercoris* present a composite spectrum containing bands at 624 and $570\text{ m}\mu$ in addition to those of the typical cytochrome components.

A tentative hypothesis that the band at $624\text{ m}\mu$ represented cytochrome a_2 was disproved by experiments, all of which indicate that neither this band nor the band at $570\text{ m}\mu$ can be due to cytochrome components. *A priori* it was unlikely that the

organisms would contain cytochrome a_2 , because this pigment has been found only in association with cytochromes a_1 and b_1 (Fujita & Kodama, 1934, Keilin, 1934, Keilin & Harpley, 1941), which are absent from the mycobacteria in question.

Successful extraction of coproporphyrin has explained the origin of the anomalous bands. In consequence of an unusually high intracellular concentration of coproporphyrin two porphyrin bands are visible, although the remainder are probably obscured by the intensity of the c and d cytochrome bands and by end absorption. The positions of the two visible porphyrin bands indicate that coproporphyrin may be present as neutral salt or as a natural ester, but not as a metal complex.

Coproporphyrin III has been isolated in the form of a tetramethyl ester, the properties of which suggest that it may be contaminated by a small amount of coproporphyrin I. It was suspected that a small quantity of coproporphyrin I (unidentified) had separated on the top of the column during chromatographic purification of the bacterial extract. Although coproporphyrin is known to occur in a variety of bacteria, yeasts and fungi (see Vannotti, 1937), isolation of characterized methyl esters has seldom been reported. Crystalline coproporphyrin esters were obtained by Fischer & Fink (1925) from *Saccharomyces ananensis* and by Kench & Wilkinson (1945), who obtained both natural isomers from brewer's yeast.

The presence of porphyrin in culture filtrates from *Corynebacterium diphtheriae* has attracted attention ever since Coulter & Stone (1931) demonstrated proportionality between toxin production and porphyrin concentration. Pappenheimer (1947) and

Pappenheimer & Hendee (1947) have shown that the relationship can depend on the iron content of the medium, and have suggested a connexion between porphyrin and cytochrome b , the chief haem pigment observable spectroscopically in suspensions of *C. diphtheriae*. Gray & Holt (1948) have isolated and characterized the porphyrin as coproporphyrin III, which is accompanied by smaller amounts of uroporphyrin I in culture filtrates.

The significance of a substantial production of coproporphyrin by some mycobacteria, and not by others, is not clear. The possibility of a connexion with cytochrome requires exploration. Preliminary experiments with *Mycobacterium Karlinski*, grown on synthetic media, indicate that specific organic nutrients are involved in coproporphyrin formation.

SUMMARY

1 The cytochrome spectrum of certain mycobacteria showing a , b and c components is complicated by two absorption bands, one at 624 $m\mu$ and the other at 570 $m\mu$. It has been shown that these bands are due to coproporphyrin.

2 Coproporphyrin III tetramethyl ester (16 mg) have been isolated from *Mycobacterium Karlinski* (4.8 kg) by an acetone extraction method. Coproporphyrin is synthesized by the organism.

3 The occurrence of coproporphyrin in other saprophytic mycobacteria has also been studied.

I wish to thank the W. H. Travis Trustees for a grant. I am also indebted to Prof. N. L. Edson for advice and guidance throughout this work, and to Miss M. Lindsay for reading the proof.

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Quantitative Studies on Mutants of *Bacterium aerogenes* Requiring Nicotinic Acid and Histidine

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(Received 25 April 1949)

In the microbiological assay of amino acids and vitamins various species of lactobacilli are the micro organisms most frequently employed. The medium used for the cultivation of the lactobacilli and of most other micro organisms at present used in assay procedure is complex, requires expensive ingredients and much time in preparation. The mutant strains of fungi such as *Neurospora* do indeed grow in simple media, but the harvesting of the mycelium is more laborious than either the turbidimetric or acidimetric measurements of bacterial growth. These disadvantages might be overcome by the use of a bacterium, in wild or mutant form, which would grow in simple medium and give a quantitative response to the required growth factor.

The assay of nicotinic acid with a strain of *Proteus vulgaris*, HX 19, was described by Grossowicz & Sherstinsky (1947). The medium contained casein hydrolysate, carbohydrate and mineral salts. The quantitative response of a mutant of *Escherichia coli* requiring arginine to the presence of arginine in simple medium and in the presence of protein hydrolysates has also recently been reported by Lampen & Jones (1947). As a preliminary study of the suitability of such mutants for use in microbiological assay the quantitative response of certain spontaneous and X ray induced mutants of *Bacterium aerogenes* (*Aerobacter aerogenes*) to nicotinic acid and to histidine has been investigated. The mutants used in the present experiments were prepared and isolated by Devi, Pontecorvo & Higginbottom (1949).

EXPERIMENTAL

Micro-organisms The mutant strains were all derived from *Bacterium aerogenes*, A10C, which grew well in a medium containing only glucose and inorganic salts. Of the two strains requiring nicotinic acid, 893 was a spontaneous mutant and 3A3 was obtained by X irradiation. Both failed to grow in simple medium in the absence of nicotinic acid. The growth requirements for nicotinic acid could be met by the corresponding amide or by relatively large amounts of quinolinic acid.

The three histidine requiring mutants were obtained by X irradiation. One, 9B2, would grow, after a lag period, in the simple medium, and has been described as an 'adaptable' mutant (Devi *et al.* 1949). The other two mutants, 297 and 506, were probably identical and failed to grow in the absence of histidine. So far as could be ascertained the growth

requirements of these mutants were specific for nicotinic acid or amide or for histidine. Stock cultures of these mutant strains were carried on agar slopes of 'complete' medium.

Media The 'complete' medium (pH 7.0) contained glucose 10 g, peptone, 10 g, yeastrel, 5.0 g and agar 15 g/l of tap water.

The 'simple' medium contained glucose 20 g, $(\text{NH}_4)_2\text{SO}_4$, 1.0 g, KH_2PO_4 , 3.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g/l. of glass distilled water. This was later modified to contain glucose, 20 g, $(\text{NH}_4)_2\text{SO}_4$, 3.0 g, KH_2PO_4 , 10.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g, sodium citrate, 18.5 g in 1 l. glass distilled water. The pH was maintained at 6.9–7.1 except where stated otherwise.

The basal (simple) medium was prepared in double the above concentration to allow for dilution by the addition of the standard solutions.

Standard solutions Stock solutions containing 200 μg /ml. were prepared by dissolving 50 mg L-histidine monohydrochloride, nicotinic acid or nicotinamide in 250 ml glass-distilled water. The stock solution of quinolinic acid contained 68 mg of the acid in 250 ml glass-distilled water and thus contained the mol. equivalent of 200 μg nicotinic acid/ml. These solutions could be stored in the refrigerator for 2 weeks without deterioration, although the solution of nicotinamide was less stable than that of the corresponding acid. From the stock solutions fresh dilutions were prepared for each experiment.

Inoculum Nicotinic acid solution (5 ml) or histidine solution (5 ml.) was added to 5 or 10 ml basal medium (depending on the micro-organism being used) and the mixture sterilized at a pressure of 10 lb/sq in. for 10 min. When cool, the solution was inoculated from a stock culture on 'complete' agar medium and incubated for 21–24 hr at 37°. The culture was then centrifuged and the bacterial sediment washed twice with 10 ml quantities of sterile quarter strength Ringer solution. The washed bacteria were suspended in 10 ml. quarter strength Ringer solution, spun at a low speed to remove clumps, and the uniform suspension suitably diluted so that the turbidity of the inoculum (read on the Spekker absorptometer) was the same in each experiment. The inoculum was freshly prepared for each assay.

Assay procedure The assays were carried out in ordinary tubes 6 in. long and $\frac{1}{2}$ in. internal diameter. Graduated doses of the standard solutions in 5 ml. were added to the tubes, 5 ml. of the basal medium (double strength) added to each tube and the contents well mixed. Triplicate tubes were set up for each dose. The tubes were then capped with inverted specimen tubes and sterilized at a pressure of 10 lb/sq in. for 10 min. When cool 0.05 ml. of the inoculum was added to each tube except the three used as controls.

After shaking well, the tubes were incubated in a water bath at 37°. Turbidimetric measurements of growth using the Spekker photoelectric absorptometer were made after

incubation for 17 hr and the results expressed as extinctions (i.e. as the Spekker drum readings). Acidimetric measurements were made after 1, 2 or 3 days incubation, using 0.1, 0.05 or 0.02 N NaOH with bromothymol blue as indicator. To each tube 1 ml of 0.04% aqueous solution of bromothymol blue was added and the contents titrated with NaOH containing 100 ml bromothymol blue/l, using a comparator.

RESULTS

Nicotinic acid requiring mutants 893 and 3A3 The response of the strain 893 to doses of 0.02, 0.04, 0.1, 0.2, 0.4 and 1.0 μ g nicotinic acid in 10 ml basal medium was determined by both acidimetric and

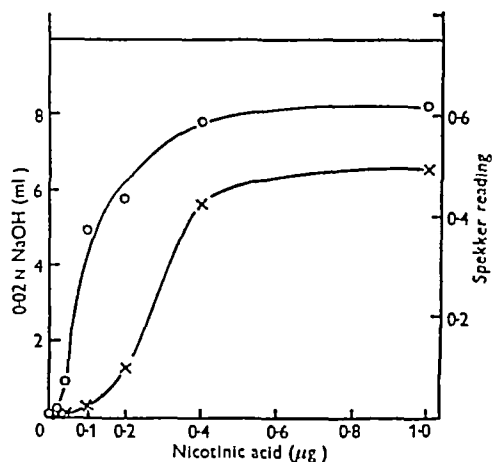


Fig. 1. Dose response curves, strain 893, to nicotinic acid in the original basal medium, $\times - \times$, turbidimetric measurements after 17 hr at 37°, $\bigcirc - \bigcirc$, acidimetric measurements after 43 hr at 37°.

turbidimetric methods. The response varied with the method of measurement (Fig. 1). The growth response to the lower doses of nicotinic acid was very poor. Thus 'lag' varied slightly in different determinations, but usually ceased with doses over 0.2 or 0.3 μ g nicotinic acid. No 'lag' was shown in acidimetric titrations.

No delay in growth or acid production was obtained with nicotinamide (Fig. 2).

The simplicity of the medium or its low buffering action may have influenced the growth response to nicotinic acid. Therefore, before further study of the mutants, various modifications of the medium, which might increase the extent of growth and acid production while still maintaining a relatively simple composition, were investigated using the parent strain.

Modifications of the basal medium The extent of growth or acid production was not appreciably affected by increasing the concentration of glucose, by the addition of 0.1% xylose or by increasing the amount of ammonium sulphate present up to 0.6%. A mixture of the purines, adenine, guanine, uracil

and xanthine, each in a concentration of 0.001%, inhibited both growth and acid production. Alanine, glycine and glutamic acid (nitrogen equivalent to that in 0.1% ammonium sulphate) did not satisfactorily replace ammonium sulphate as the source of nitrogen, although the addition of asparagine or glutamic acid to the basal medium gave increased growth and acid production.

By the addition of 0.75% vitamin free casein hydrolysate (Ashe Laboratories Ltd.) to the basal medium, the growth as measured turbidimetrically was doubled, although acid production was only increased by one third. The responses were similar,

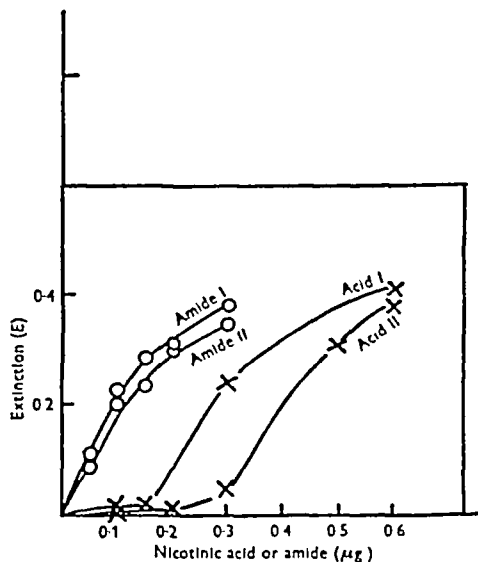


Fig. 2. Dose response curve, strain 893, to nicotinic acid and amide in original basal medium, turbidimetric measurements (Exps. I and II) after 17 hr at 37°.

but much less marked, with a mixture of lysine and glutamic acid in the amounts presumed to be present (Schmidt, 1945) in 0.75% casein hydrolysate. Proline inhibited both growth and acid production, but this inhibition was removed by the addition of lysine.

To counter the poorer acid production compared with growth in the presence of casein hydrolysate, modification of the buffering agents was investigated. Sodium acetate (0.16–4.0%) or the corresponding amounts of potassium acetate inhibited growth and acid production. The concentration of potassium dihydrogen phosphate was increased by two, three, four and six times that in the original basal medium. The greater the phosphate content the greater the amount of growth and particularly the acid production (titratable acidity). This effect was slightly enhanced by increasing the amount of glucose and ammonium sulphate to 3 and 0.3% respectively (Table 1).

Table 1 *The effect of addition of increased amounts of phosphate and other substances on growth and acid production by Bact aerogenes A10C*

(Media A, B, C and D contained respectively two, three, four and six times the amount of KH_2PO_4 in the basal medium)

Medium	Additions (%)	Turbidimetry after 17 hr at 37° (Extinction E)	Acidimetry after 43 hr at 37° (ml 0.05N NaOH)
A	Nil	0.486	9.5
	$(\text{NH}_4)_2\text{SO}_4$, 0.2	0.453	9.7
	Glucose, 1	0.463	9.8
	$(\text{NH}_4)_2\text{SO}_4$, 0.2 + glucose, 1	0.518	10.1
B	Nil	0.570	13.0
	$(\text{NH}_4)_2\text{SO}_4$, 0.2	0.663	13.7
	Glucose, 1	0.555	14.0
	$(\text{NH}_4)_2\text{SO}_4$, 0.2 + glucose, 1	0.645	14.3
C	Nil	0.691	15.6
	$(\text{NH}_4)_2\text{SO}_4$, 0.2	0.720	16.8
	Glucose, 1	0.650	16.5
	$(\text{NH}_4)_2\text{SO}_4$, 0.2 + glucose, 1	0.661	17.6
D	Nil	0.733	23.3

Although the highest growth response was obtained with a four to six-fold increase in phosphate, discoloration of the medium occurred during sterilization. To avoid the necessity of sterilizing the

reaction in the various modifications of the medium was always adjusted to between pH 6.9 and 7.1.

The final modified basal medium, the composition of which was given on p. 391, contained increased amounts of ammonium sulphate and potassium dihydrogen phosphate and in addition sodium citrate. This basal medium was used for all subsequent

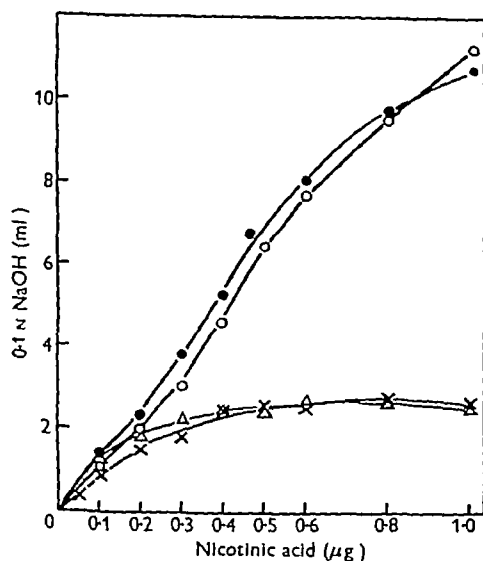


Fig 3 Dose-response curve, strains 893 and 3A3, with nicotinic acid in original and modified basal medium. Acidimetric measurements after 48 hr at 37°, x—x, strain 893 in original medium, o—o, strain 893 in modified medium, Δ—Δ, strain 3A3 in original medium, ●—●, strain 3A3 in modified medium.

glucose and phosphate portions of the medium separately, a threefold increase in phosphate was adopted. Additional buffering associated with a further increase in both growth and acid production was obtained when potassium (2%) or sodium (1.85%) citrate was included in the medium with three times the original amount of phosphate. The

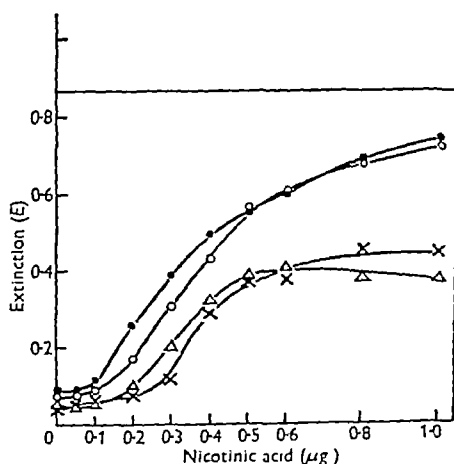


Fig 4 Dose response curve, strains 893 and 3A3, to nicotinic acid in original and modified basal medium. Turbidimetric measurements after 17 hr at 37°, x—x, strain 893 in original medium, o—o, strain 893 in modified medium, Δ—Δ, strain 3A3 in original medium, ●—●, strain 3A3 in modified medium.

quantitative studies of the mutant strains of *Bact aerogenes*.

Quantitative response to nicotinic acid and amide
The dose response curves of strains 893 and 3A3 to nicotinic acid in the original and the modified basal medium are given in Fig 3 (titratable acidity) and Fig 4 (turbidity measurement). The maximum

response, whether measured by acidimetric or turbidimetric methods, was greatly improved by the use of the modified medium, but the 'lag' in growth response to small doses of nicotinic acid in the turbidity method remained.

The addition of 0.3% vitamin free casein hydrolysate to the modified basal medium did not affect the 'lag' in response to nicotinic acid (Fig 5). Indeed the response was rather less in the presence than in the absence of casein hydrolysate except at the higher dose levels. The growth response to nicotinamide was greater at all dose levels in the presence of casein hydrolysate.

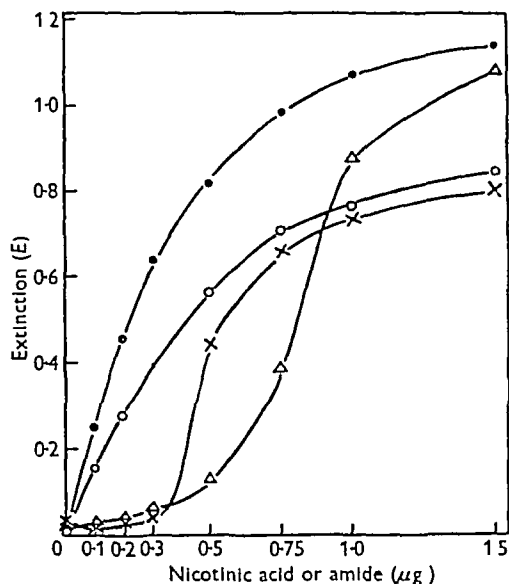


Fig 5 Dose response curve, strain 893, with nicotinic acid and nicotinamide in modified basal medium with and without casein hydrolysate. Turbidimetric measurements after 17 hr at 37°, x—x, nicotinic acid without casein hydrolysate, O—O, nicotinic acid with casein hydrolysate, Δ—Δ, nicotinamide without casein hydrolysate, ●—●, nicotinamide with casein hydrolysate.

Factors affecting the 'lag' in growth response In turbidimetric estimations of growth the incubation time was shorter, and Spekter readings were less time consuming than the acidimetric estimations. Therefore in an attempt to eliminate the 'lag', and thus perfect the turbidimetric measurements of response, the effect of size of inoculum, reaction of the medium and the length of the incubation period at 37° were studied.

Increase in the density of the inoculum tended to reduce the 'lag' somewhat and also increased the growth response to higher doses of nicotinic acid. The reaction of the medium had a definite effect on the response. At pH 6.0 or below the 'lag' was eliminated, but under such conditions the response

to higher doses of nicotinic acid was much less than at pH 7.0 (Fig 6). These results stress the importance of careful control of the reaction of the medium. The 'lag' was markedly reduced or eliminated by increasing the incubation period at 37° from the usual

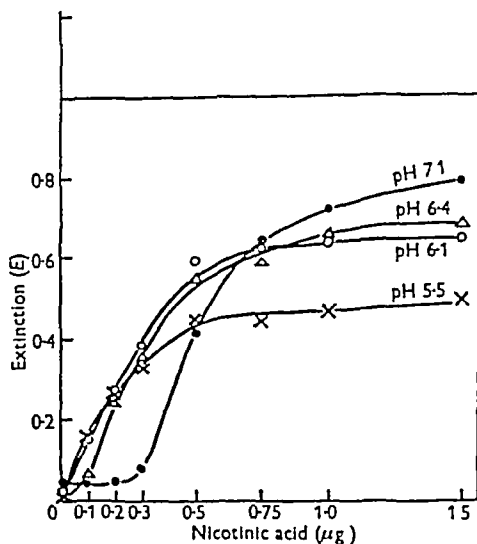


Fig 6 Effect of hydrogen ion concentration on the dose response curve of strain 893 with nicotinic acid, turbidimetric measurements after 17 hr at 37°.

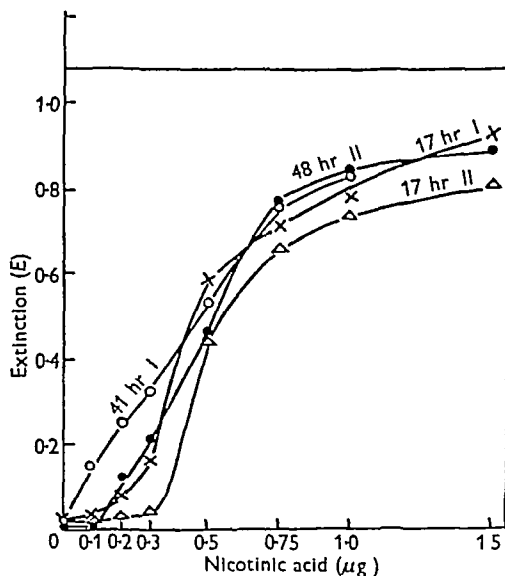


Fig 7 Effect of period of incubation at 37° on dose response curve of strain 893 with nicotinic acid, turbidimetric measurements (Exps I and II).

17 hr to 41 or 48 hr (Fig 7). The absence of a 'lag' in the response measured by titration may thus have been due to the use of the longer incubation time of 48 hr. Finally, since there was no 'lag' in response

to the amide, an addition of nicotinamide to give a final concentration of $0.05 \mu\text{g}$ /assay tube was made to the modified basal medium. The growth response of strains 893 and 3A3 to nicotinic acid showed no 'lag' in the presence of suboptimal amounts of nicotinamide (Fig 8)

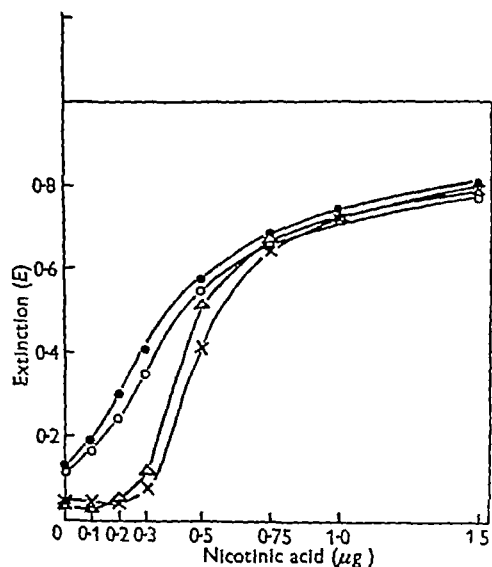


Fig 8 Dose-response curves, strains 893 and 3A3, with nicotinic acid in modified medium with and without suboptimal amounts of nicotinamide. Turbidimetric measurements after 17 hr at 37° , x—x, strain 893 without amide, o—o, strain 893 with amide, Δ—Δ, strain 3A3 without amide, ●—●, strain 3A3 with amide

Response to quinolinic acid No growth was obtained with quinolinic acid in doses equivalent to 0.1 – $1.5 \mu\text{g}$ nicotinic acid. With doses of quinolinic acid about 1000 times that of nicotinic acid a response similar to that with nicotinic acid was obtained. It seems likely that this response was due, not to quinolinic acid itself, but to nicotinic acid present as an impurity or formed during the heat sterilization of the quinolinic acid. Similar results were obtained by Koser, Dorfman & Saunders (1940) with strains of *Proteus* sp. and dysentery bacilli which required nicotinic acid.

The histidine requiring strains 297, 506 and 9B2 The response of strains 297 and 506 to 0, 10, 20, 30, 40, 50, 75, 100, 125 and $150 \mu\text{g}$ histidine in the modified medium is given in Fig 9. A smooth curve was given by strains 297 and 506 indicating a quantitative response of both growth and acid production to graded doses of histidine. The response with strain 9B2, an 'adaptable' mutant, was much greater, $10 \mu\text{g}$ of histidine giving as much growth as $125 \mu\text{g}$ histidine with strains 297 and 506.

The dose response curves of strain 9B2 with histidine in doses from 0 to $30 \mu\text{g}$ are given in

Fig 10. The acidimetric titrations in this instance were recorded after incubation for 19 hr when about three times as much acid was produced as after incubation for 43 hr with strains 297 and 506.

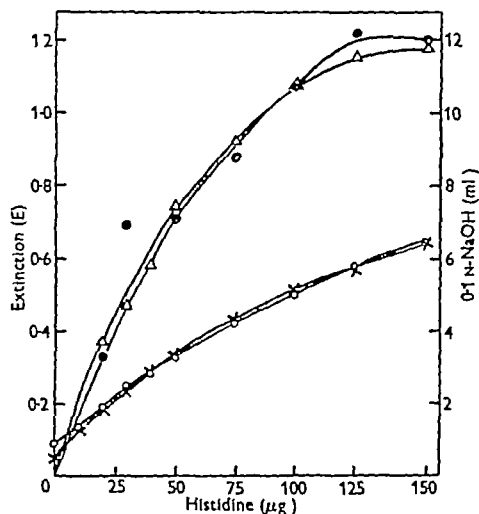


Fig 9 Dose response curve, strains 506 and 297, with histidine. Turbidimetric measurements after 17 hr at 37° , x—x, strain 506, o—o, strain 297. Acidimetric measurements after 43 hr at 37° , Δ—Δ, strain 506, ●—●, strain 297

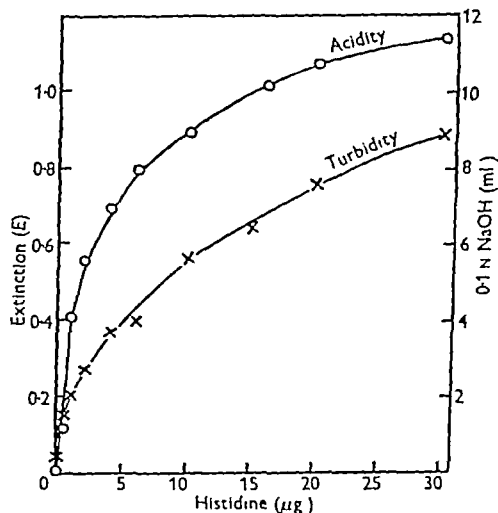


Fig 10 Dose response curves of strain 9B2 to histidine, x—x, turbidimetric measurements after 17 hr at 37° , o—o, acidimetric measurements after 19 hr at 37°

DISCUSSION

The behaviour of the nicotinic acid requiring mutants of *Bact. aerogenes*, designated 893 and 3A3, has conformed to that previously described for similar mutants of other micro organisms. A lack of growth

response, a 'lag', in the presence of low doses of nicotinic acid has been observed by Bonner & Beadle (1946) with nicotinic requiring mutants of *Neurospora*. With mutants of *Esch. coli*, Roepke, Libby & Small (1944) found a close correlation between the activity of nicotinic acid at a given pH and the dissociation value of the acid. The dissociation of nicotinic acid decreased with decrease in hydrogen-ion concentration, and it was concluded that only the undissociated acid could enter the cell and be utilized for metabolic purposes.

On the other hand, the relationship between the response to nicotinic acid and the corresponding amide showed a very close parallel with the results obtained in the utilization of glutamic acid and glutamine by *Lactobacillus arabinosus* (plantarum) (Lyman, Kuiken, Blotter & Hale, 1945; Hac, Snell & Williams, 1945). As in the present investigation with nicotinic acid and amide, the lag in response to small doses of glutamic acid was not obtained with the corresponding amide. Hac *et al.* (1945) showed that the 'lag' tended to be minimized by increasing the size of the inoculum, by longer periods of incubation and by an acid reaction in the medium. Lyman *et al.* (1945) found that the 'lag' with the acid could be eliminated by the addition of minimal amounts of the amide to the basal medium. They concluded that their results could readily be explained by the assumption that glutamine, rather than glutamic acid, was the substance actually utilized by the micro organisms. Similarly, it might be concluded that nicotinic acid must be converted to nicotinamide before it can be utilized by the mutants 893 and 3A3. The most important part played by nicotinic acid is in relation to the phosphopyridine nucleotides where it is present as the amide. This would appear to give further support to the suggestion that the first step in the utilization of nicotinic acid is conversion to the amide.

The histidine requiring strains gave a quantitative growth response to doses of 0–150 μ g of histidine. The 'adaptable' mutant, 9B2, proved very much more sensitive in its response to the histidine. This may be related to its ability to grow in the basal medium in the absence of added histidine after a delay of at least 24 hr.

The application of these mutants to the actual assay of nicotinic acid or amide and of histidine in

biological material has not yet been attempted. The non specific effect of the presence of other substances has not been studied with the mutants requiring histidine. The addition of casein hydrolysate to the basal medium did produce a further increase in growth response to nicotinic acid or amide, and it seems likely that further modification of the basal medium would be required should these mutants be used in microbiological assays.

SUMMARY

1 A study has been made of the quantitative response of certain mutants of *Bacterium aerogenes* (*Aerobacter aerogenes*), which require nicotinic acid and histidine, to the required metabolite in a simple basal medium containing glucose and mineral salts. The effect of making various modifications in the mineral salt content of the medium was investigated.

2 The strains requiring nicotinic acid, after incubation for 17 hr, showed a 'lag' in growth response to doses of nicotinic acid under 0.2–0.3 μ g, but not to nicotinamide. The limiting doses at which the 'lag' in growth response ceased varied somewhat in different estimations.

3 The 'lag' was reduced by increasing the size of the inoculum, by lengthening the period of incubation or by increasing the acidity of the medium. It could be eliminated by the addition of minimal amounts of nicotinamide to the basal medium.

4 A quantitative response to histidine in doses from 0 to 150 μ g was obtained with the non-adaptable histidine requiring mutants. The 'adaptable' mutant showed a much greater response to histidine.

5 The possibility of the use of these mutants in microbiological assay is briefly discussed.

The authors wish to thank Dr E. R. Dawson and his staff at the Research Laboratories of the Distillers Co. Ltd., Great Burgh, Epsom, where facilities were provided for experience in the technique of microbiological assay. The authors also wish to thank Dr G. Pontecorvo of the Department of Genetics in the University of Glasgow, at whose suggestion the study of mutant strains of bacteria was begun and with whose collaboration the mutants used in the present work were isolated. One of us (P. D.) wishes gratefully to acknowledge the tenure of a Scholarship from the Government of India.

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The Composition of Foetal Fluids of Sheep at Different Stages of Gestation

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(Received 1 April 1949)

One of the interesting problems of foetal physiology is the origin, function and interrelation of the allantoic and amniotic fluids. Some information can be obtained by a study of the composition of the two fluids. Most of our knowledge on the subject, in the case of ruminants, is provided by the papers of Jacque (1902), Paton, Watson & Kerr (1907) and Malan, Malan & Curson (1937) on sheep, and of Doderlein (1890) on cows. The older work refers to unspecified material, usually obtained from a slaughterhouse and arranged only according to foetal length or weight. The results of Malan *et al.* (1937) refer to Merino ewes at given stages in gestation and are the most complete. The present work reports the composition of a series of foetal fluids which had been obtained from cross bred ewes of defined stages in gestation and management during pregnancy. The properties and constituents studied are the specific gravity, dry matter, ash, total and non-protein nitrogen, pH and principal anions and cations of physiological interest.

EXPERIMENTAL

Material. Twelve samples of allantoic and thirteen of amniotic fluid were obtained from some of the ewes in the experiments of Wallace (1948) on the growth of lambs before and after birth. The animals were his 'age series' of ewes. These were of Border Leicester \times Cheviot breed and had been mated with a Suffolk ram. They were fed a standard ration of sanfoin hay and concentrates, which was sufficient to meet requirements for maintenance and reproduction, without fattening the ewe appreciably by the end of pregnancy. They were killed in groups at 28 day intervals during the gestation period.

Treatment of samples. Immediately after slaughter of the ewes, the uterus and contents were removed, the fluids put into beakers and kept in a cold store. Samples for analysis were usually taken within a few days, and stored in waxed tubes with a crystal of thymol at 4° until they could receive attention. The last samples of allantoic fluid contained suspended solid matter, presumably meconium. This was allowed to settle and the supernatant fluid only taken for analysis.

Chemical methods. Specific gravity was determined as the ratio of the weights (to the nearest mg.) of fluid and water in a 10 ml. density bottle at room temperature (17–20°). Dry matter, ash and organic matter were determined by drying 2 ml. of the fluid to constant weight at 110° in

a porcelain crucible, ashing as in the Stolte process, described by Peters & Van Slyke (1932), and weighing again.

N was estimated by micro Kjeldahl, total N on 0.5 ml. allantoic fluid and 1.0 ml. amniotic fluid, and non-protein N on 5.0 ml. filtrate from a mixture of 2.5 ml. fluid and 10 ml. of 10% (w/v) trichloroacetic acid solution, protein N was calculated by difference. In some samples, notably the later ones of amniotic fluid, a clear trichloroacetic acid filtrate could not be obtained, in which case the results are incomplete.

Inorganic ions were estimated by standard methods used in serum analysis as follows: Na (Kramer & Gittleman, 1924), K (Kramer & Tisdall, 1921) precipitation followed by solution in excess ceric sulphate and back titration with 0.02N $\text{Na}_2\text{S}_2\text{O}_8$ after adding KI, Ca (Clark & Collip, 1925), Mg (Denis, 1922), chloride (Sendroy, 1937), inorganic P (Fiske & Subbarow, 1925), pH was determined with a quinhydrone electrode and the CO_2 combining capacity in a volumetric Van Slyke apparatus, after equilibrating with alveolar air.

These methods were applied as follows: Ca, Mg, chloride and P were estimated directly on both fluids, and Na directly on the amniotic fluid. Na in the allantoic fluid and K in both fluids were estimated on the ash obtained by the Stolte process (see Peters & Van Slyke 1932), as direct estimation gave erratic results, when insufficient sample remained to repeat the analysis on the ash, the results are incomplete. Suitable dilutions of the sample were made for the determination of K, Ca, and Mg in the allantoic fluid. In estimating P in the allantoic fluid, difficulty was encountered with samples obtained after the first 2 months of pregnancy, as the values were low and a turbidity developed on the addition of the molybdic sulphuric reagent, however, an approximate value was obtained by extracting the molybdenum blue with isoamyl alcohol and comparing with a similarly treated standard.

RESULTS

The results of analyses are given in Table I, together with details of the samples. The allantoic fluid showed an increase, as gestation proceeded, in specific gravity, non-protein nitrogen and other organic matter, potassium and magnesium, and a decrease in chloride and inorganic phosphorus, the values for calcium were higher in the middle than at the beginning or end of gestation. The amniotic fluid, on the other hand, was much more constant in composition. It had a lower specific gravity, lower organic matter, non-protein and protein nitrogen and magnesium contents, and higher sodium and chloride contents, than the allantoic fluid. The potassium, calcium and phosphorus contents fell within the range of values of this fluid.

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Table 1 The composition of foetal fluids of sheep at various stages of gestation

Stage (days)*	No of foetus*	Wt of foetus (g)*	Sex	Quantity of fluid (ml)*	Sp gr	Dry matter		Ash	Organic matter (g/100 ml)	Total N	Protein N			pH	P					CO ₂ combining capacity (ml/100ml)
						N	P				N	Na ⁺	K ⁺		Ca ⁺⁺	Mg ⁺⁺ (mg/100 ml)	Cl ⁻ (mg/100 ml)	(inorganic)		
Allantoic fluid																				
28	6	0 47	—	32	1 0082	0 85	0 6	0 25	0 118	0 086	0 032	8 00	—	47	9 7	3 7	230	10 4	102	
28	8a	0 48	—	18 8	—	0 75	0 6	0 15	—	—	—	8 00	177	21	12 0	2 9	240	13 0	60	
56	3b	47	—	93	1 0106	1 8	0 45	1 35	0 214	0 185	0 029	7 31	163	20	11 9	6 4	240	4 8	31	
56	10	46	—	74	1 0156	2 3	0 6	1 7	0 315	0 210	0 105	6 87	139	38	9 3	8 7	249	7 4	9	
84	11a	525	M	74	1 0186	—	—	—	0 350	0 287	0 063	6 89	—	—	24 4	22 9	80	0 9	15	
84	11b	486	M	59	1 0174	1 1	0 8	0 3	0 343	0 236	0 107	7 36	—	—	20 7	19 3	113	0 4	22	
84	9b	490	F	49	—	—	—	—	0 770	0 546	0 224	6 36	—	—	6 3	19 0	51	<1	—	
112	14	1624	F	308	1 0204	4 3	1 25	3 05	0 323	0 280	0 043	4 49	—	40	40 0	34 1	43	<1	—	
112	12a	1985	F	248	1 0218	—	—	—	0 404	0 368	0 036	—	—	—	—	—	—	0 4	—	
112	12b	2248	ML	422	1 0205	4 15	1 05	3 1	0 374	0 330	0 044	5 14	202	119	9 5	41 0	70	0 5	—	
140	7	6226	M	1050	1 0233	3 9	1 15	2 75	0 360	0 350	0 010	6 46	266	141	4 6	79	42	0 5	15	
140	15	5925	F	1167	1 0202	3 7	1 3	2 4	0 304	0 293	0 011	6 64	86	330	2 5	30 5	40	—	10	
Amniotic fluid																				
28	6+2	0 47+0 55	—	0 85+0 87	—	—	—	—	0 08	0 08	0 00	—	—	—	—	—	—	—	—	
28	8a+b	0 48+0 55	—	1 19+1 19	—	—	—	—	—	—	—	—	302	—	—	—	378	—	—	
56	3b	47	—	164	1 0074	—	—	—	0 038	0 031	0 007	7 17	206	67	7 1	1 4	438	2 8	47	
56	3a	42	—	184	1 0074	1 15	0 9	0 25	0 038	0 033	0 005	7 47	263	57	6 3	1 3	446	2 7	44	
56	10	46	—	149	1 0082	1 0	0 8	0 2	0 061	0 038	0 023	7 39	258	75	6 6	2 2	109	6 1	35	
84	11a	525	M	630	1 0074	—	—	—	0 034	0 028	0 006	7 67	309	—	6 9	1 5	424	1 5	45	
84	11b	486	M	675	1 0066	1 15	0 8	0 35	0 032	0 029	0 003	7 60	312	37	7 0	1 6	432	1 3	48	
84	9b	490	F	602	1 0070	—	—	—	0 035	0 025	0 010	7 12	302	—	6 7	1 4	447	2 0	37	
112	14	1624	F	255	1 0079	1 35	0 85	0 55	0 076	—	—	6 68	287	—	10 8	2 8	448	0 7	—	
112	12a	1985	F	738	1 0071	1 5	0 9	0 6	0 055	—	—	4 86	293	53	9 2	3 0	430	3 0	—	
112	12b	2248	M	1263	1 0077	1 5	1 15	0 35	0 060	—	—	4 53	284	47	8 2	2 8	416	2 5	—	
140	7	6226	M	1684	1 0078	1 2	0 75	0 45	0 072	—	—	8 40	279	43	5 9	3 4	382	1 3	67	
140	15	5925	F	1416	1 0075	1 1	0 65	0 45	0 073	—	—	6 40	233	33	4 3	2 6	348	1 5	18	

* Data quoted from Wallace (1948)

DISCUSSION

Before discussing the results, it is appropriate to consider possible effects of the treatment of the experimental material in terms of conditions and processes obtaining in the womb of the living ewe. Post mortem changes may set in before the removal of the fluids from the foetal sacs. These might alter the composition through differences in the permeability of the membranes separating the two fluids, or in the functioning of the foetal kidney. The possibility of such changes was emphasized by Jacque (1902), but not explicitly avoided by others. They are considered to be negligible in the present work.

After taking the samples there may be a loss of dissolved gases. Should the fluid contain any appreciable amount of carbon dioxide, the pH value and carbon dioxide content would then both be affected. In the present work, samples could not be obtained anaerobically and so the carbon dioxide combining capacity, rather than the carbon dioxide content, was determined. The pH values reported may consequently be too high. Reliable figures for pH and carbon dioxide content have, therefore, yet to be reported.

During storage of the samples, enzymic changes might occur. Any due to micro organisms were avoided in the present work. It is not known, however, whether the fluids themselves contain enzymes derived from the foetus. As they are known to contain fructose the possibility of changes, which might affect the acidity, remains. This might have the additional effect in those samples of allantoic fluid, which contained suspended solid matter, of bringing more ions into solution.

The present results are mostly in general agreement with those already reported in the literature, but the following differences may be noted. Malan *et al* (1937) reported a number of much lower values for the specific gravity, especially of the amniotic fluid. They also found higher values for the phosphorus in the allantoic fluid in the latter part of gestation, but it is not clear whether their results refer to the inorganic, as in the present work, or to the total phosphorus. Doderlein (1890) gives some significantly higher values for the calcium in the amniotic fluid.

The changes in the non-protein nitrogen, organic matter, potassium and calcium contents of the allantoic fluids suggest an increasing excretion of foetal urine as gestation proceeds. This implies that the composition of the allantoic fluid reflects the developing metabolic activity of the foetus, and the developing functional efficiency of its kidney. The decreased chloride in the 84 day samples, and the increased volume of the 115-day samples may therefore indicate the appearance of tubular activity. Although little is known of the composition of

ruminant foetal urine, the limited data of Doderlein (1890) and Jacque (1902) support the view that urinary excretion does take place. Jacque (1902), however, reached somewhat different conclusions as to the routes of excretion on the basis of his extensive cryoscopic investigations. He considered that urine was excreted successively via the urachus alone, the urachus and urethra and finally the urethra alone, as gestation proceeded. The present results, however, may not necessarily be incompatible with this. Urinary excretion may not follow a rigid pattern in a given species, and may possibly be affected by variable factors, such as the management of the ewe during pregnancy.

Table 2 Comparison of average compositions of sheep amniotic fluid and serum

	Amniotic fluid		Serum*
	(mg /100 ml.)	(m equiv /l.)	(m equiv /l.)
Na ⁺	287	124.8	158.6
K ⁺	51.5	13.1	8.9
Ca ⁺⁺	7.2	3.6	5.2
Mg ⁺⁺	2.2	1.8	2.8
Total cations	—	143.3	175.5
Cl ⁻	416.5	117.3	104.2
P (inorganic)	2.3	about 1.0	1.7
CO ₂	46.3	20.7	25.0
Total anions	—	139.0	130.9

* Based on data in Shearer & Stewart (1931) and Dukes (1943)

The results for the amniotic fluid justify the calculation of an average composition (cf Table 2). When expressed as m-equiv/l the sums of the anions and cations agree to $\pm 1.5\%$, indicating that the analyses for the main ionic constituents are fairly complete. Inspection of the data suggests comparison with the composition of sheep serum, and an average composition for this fluid, based on the literature, has been included in Table 2. The amniotic fluid is seen to have a slightly lower sodium and higher chloride content, and a calcium content nearer to the ionizable than to the total calcium of serum. This suggests that the amniotic fluid may be a transudate of serum. The possibility may be sufficiently tested for the present, by considering the relations of the predominant ions, sodium and chloride, in the two fluids. The conditions for a Donnan equilibrium are found to hold to $\pm 6\%$. To establish this view more definitely, concurrent analyses of amniotic fluids, and maternal and foetal sera from the same uteri, are required. That sodium can readily pass from the maternal serum to the amniotic fluid has been shown for rats and guinea pigs by the radioactive tracer studies of Flexner & Gellhorn (1942). A similar permeability in the structurally different placenta of the sheep would at least allow the possibility of an ionic equilibrium between the two fluids.

A direct relation between the amniotic and allantoic fluids across the placental membranes seems improbable from the present results. Therefore, if the amniotic fluid is absorbed by the foetus, the foetal digestive tract and kidney would be the main agents determining the quantity and composition of the allantoic fluid. Hence the present 'passive' study of the composition of the two fluids gives us the following picture of their origin and interrelation: the amniotic fluid arises as a transudate of the maternal serum and the allantoic fluid comes from the amniotic fluid by the intervention of the foetus. This view could be tested further by a combination of the more active experimental approach of some of the earlier workers with present day techniques, and would need to be correlated with both physiological and anatomical evidence.

SUMMARY

1 The composition, mainly ionic, of a series of allantoic and amniotic fluids obtained, at five regular intervals during gestation, from ewes fed during pregnancy to maintain their weight to the end of pregnancy, has been studied.

2 As gestation proceeded the allantoic fluid showed an increase in non protein nitrogen and other organic matter, potassium and magnesium, and decrease in chloride and inorganic phosphorus. The values for calcium were higher in the middle than at the beginning or end of gestation.

3 The amniotic fluid was more constant in composition, it had a lower specific gravity and organic matter, non protein and protein nitrogen and magnesium contents, and higher sodium and chloride contents than the allantoic fluid. Potassium, calcium and inorganic phosphorus contents were within the ranges for allantoic fluid.

4 Comparison of these results, with data available for the composition of sheep serum and foetal urine, suggest that the amniotic fluid is in Donnan equilibrium with the maternal serum, and that the chemical differences between the amniotic and allantoic fluids are due to the intervention of the digestive tract and kidney of the foetus.

I am indebted to Dr L. R. Wallace, Animal Research Station, University of Cambridge, for the supply of foetal fluids and for permission to include in Table 1 some of his data about the fluids.

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A Photoelectric Flame Photometer

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(Received 29 April 1949)

The flame photometer is an instrument for determining the concentrations of certain metals in solution by measuring the intensity of the light emitted by them when the solution is sprayed under controlled conditions into a non luminous flame. Lundegårdh (1929-34) developed an apparatus in which cations in solution were sprayed into an air-acetylene flame, and the spectra produced photographed and compared with similar spectra from

standard solutions. More recently several workers (for literature, see Barnes, Richardson, Berry & Hood, 1945; Boon, 1945) have designed instruments in which the light from the flame is passed through optical filters on to a photosensitive element (selenium cell or phototube) and the current so produced is measured. Such instruments may be bought in the United States (from the Perkin-Elmer Corporation, Glenbrook, Connecticut, or the National

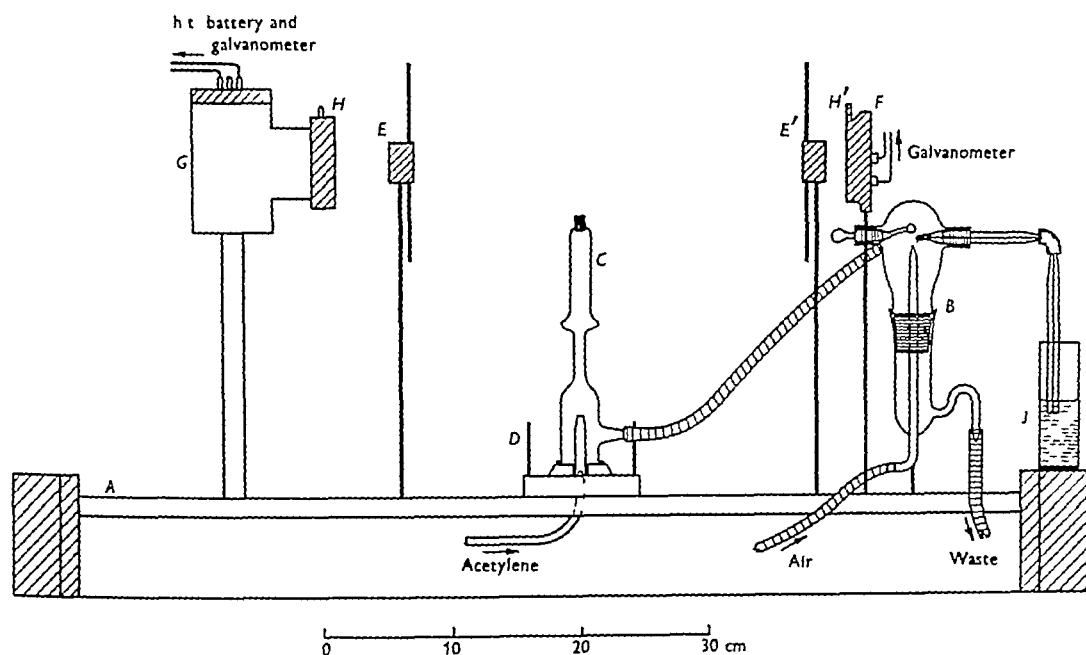


Fig 1 Flame photometer, general arrangement, excluding chimney, gas cylinders, manometers and galvanometer
A, optical bench, *B*, atomizer, *C*, burner, *D*, support for chimney, *E*, *E'*, iris diaphragms, *F*, selenium cell in holder, *G*, phototube in holder, *H*, *H'*, optical filters, *J*, solution under test

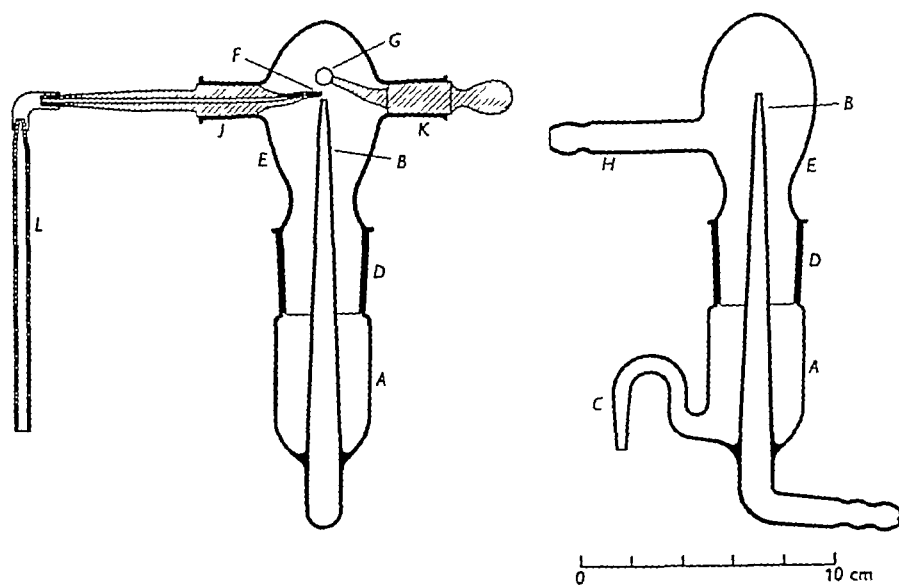


Fig 2 Atomizer (two vertical cross sections at right angles) *A*, lower part carrying air inlet (*B*) and drain for waste (*C*) *A* is joined by the ground glass joint (*D*) to the upper part *E*, which carries the solution inlet (*F*), the baffle plate (*G*) and the outlet to the burner (*H*) *F* and *G* are mounted on ground glass joints (*J*, *K*) *F* is joined by a piece of narrow rubber tubing to the vertical tube *L* which dips into the solution to be analysed.

Technical Laboratories, South Pasadena, California), but are difficult to obtain in Europe. The instrument described below can be constructed from parts which are available from stock by any laboratory which has a good mechanic. Its design is based on that of Boon (1945), but, since the latter described his instrument only in a dissertation which is not generally accessible, it was thought desirable to give this account of the construction of our apparatus and its use for the determination of sodium and potassium in biological fluids. The use of the instrument in agricultural chemistry will be described by one of us (W R D) elsewhere.

Preliminary accounts of this work have already been published (Domingo, Klyne & Weedon, 1948, Klyne, 1948, 1949).

CONSTRUCTION

The apparatus consists essentially of an optical bench—carrying an atomizer, a burner, a selenium cell and a photo tube with appropriate optical filters (Fig 1)—gas cylinders, manometers and a galvanometer.

Gas supply and regulation

Air and acetylene are supplied from cylinders carrying two stage regulators (British Oxygen Co. Ltd), which regulate the pressures to 10 lb/sq in for air and 6 lb/sq in for acetylene. Each gas is carried from the cylinder through reinforced rubber tubing (5 mm internal diameter) to a glass tap (1.5 mm bore), which is rotated by a slow motion dial to adjust the gas flow more precisely. (The slow motion dials are of the type frequently used in radio equipment.) Beyond the glass tap each gas passes to a T piece, one arm of which is connected to the apparatus whilst the other is connected to a U tube manometer (height 55 cm). An air pressure of 40 cm mercury and an acetylene pressure of 38 cm water have been found convenient with this apparatus, these pressures are dependent on the size of the air and solution inlets. The glass taps, slow motion dials and manometers are all conveniently mounted on a vertical board beside the optical bench.

Atomizer

The all glass atomizer (Fig 2) resembles that of Rautenberg & Knippenberg (1940), and can be obtained from the Laboratory Glassblowers Co., 63 Lowlands Road, Harrow, Middlesex. Air flowing up the tube *B* past the tip of the solution inlet *F* creates sufficient suction to draw solution up the tube *L* and along *F* and then blow it into a fine spray. The larger particles of this spray strike the plate *G* and are retained, the finer particles pass through *H* and thence to the burner.

Burner

The glass burner (Fig 3) resembles that of Lundegårdh (1929–34, cf McClelland & Whalley, 1941, Mitchell, 1948) and can be obtained from the Laboratory Glassblowers Co., Harrow. The dimensions of the atomizer and burner only are critical, the dimensions of the remaining parts of the apparatus can probably be varied somewhat without greatly

affecting its performance. The burner is held in position by the metal support (Fig 4) and is surrounded by a metal chimney (Fig 5) carrying two holes for a condenser lens and an inspection hole. The centre line of the lens holes is 3.5 cm above the tip of the burner, and 23.5 cm above the base of the burner.

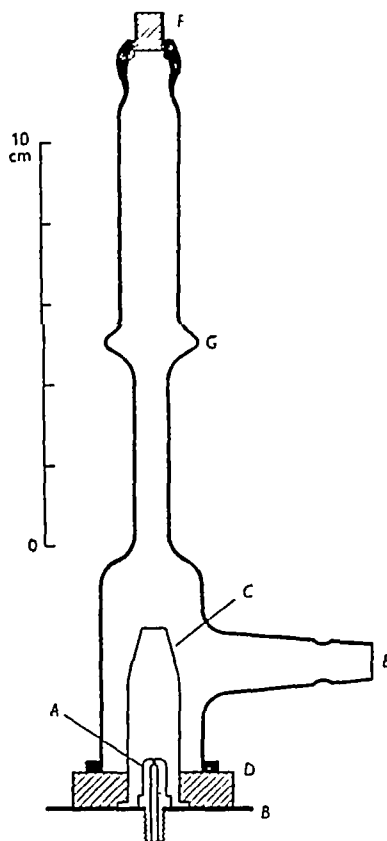


Fig 3 Burner (vertical section) *A*, acetylene inlet (0.5 mm diameter) screwed into base plate *B*, *C*, inner cone fitting into rubber washer (*D*), *E*, inlet for air carrying spray from atomizer, *F*, platinum tip, *G*, wider part of tube to fit into support.

Optical and electrical system

The base of the burner and chimney is mounted on an optical bench, one end of which is used for Na determinations and the other for K determinations.

Determination of sodium (Fig 6) The light from the outer cone of the flame *C* passes through a plano convex lens *E*, the iris diaphragm *F* and the optical filters *H* on to a selenium cell *J* (type A, 25 mm diameter, Evans Electro Selenium Ltd, Harlow, Essex). The Chance orange filter (no OY1) with an Ilford blue filter (no 803) has proved suitable for isolating the Na *D* lines (589 and 590 mμ). The current from the selenium cell is fed directly on to a galvanometer, a Tinsley SS5 box galvanometer (deflexion 85 mm/μA) is convenient. We have used a reciprocal logarithmic scale of the type commonly employed on photoelectric colorimeters (cf King, 1947) graduated in terms of $y = 100 \log (100/x)$.

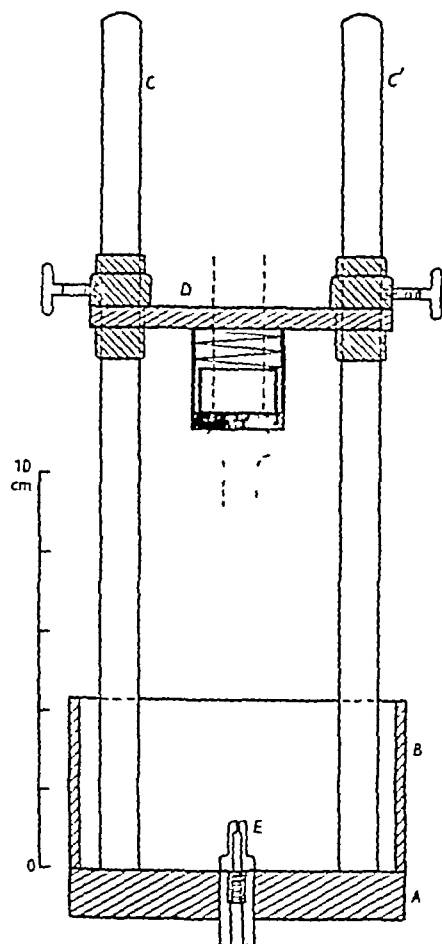


Fig 4. Support for burner (vertical section) *A*, base plate, *B*, support for chimney with seven air holes (25 mm diameter), *C*, *C'*, supports, *D*, spring loaded collar fitting on to wider part of burner (shown in dotted lines), *E*, acetylene inlet

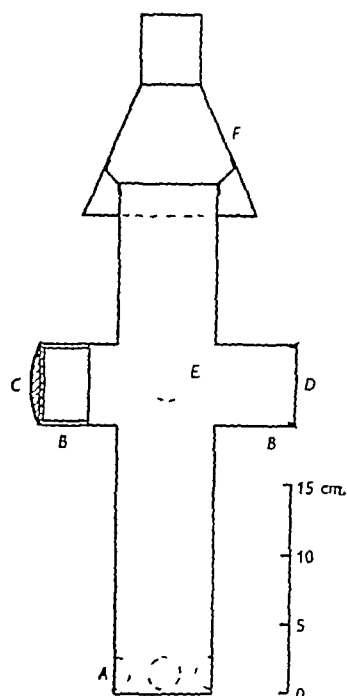


Fig 5. Chimney (vertical section) *A*, base with holes (each 25 mm in diameter) to admit air (fits into support Fig 4 *B*), *B*, *B'*, holes for lens, *C*, plano convex lens (focal length 9 cm.) in sliding tube, *D*, metal plate to cover hole not used for lens, *E*, shows the position of the inspection hole on the front of the chimney, *F*, cowl.

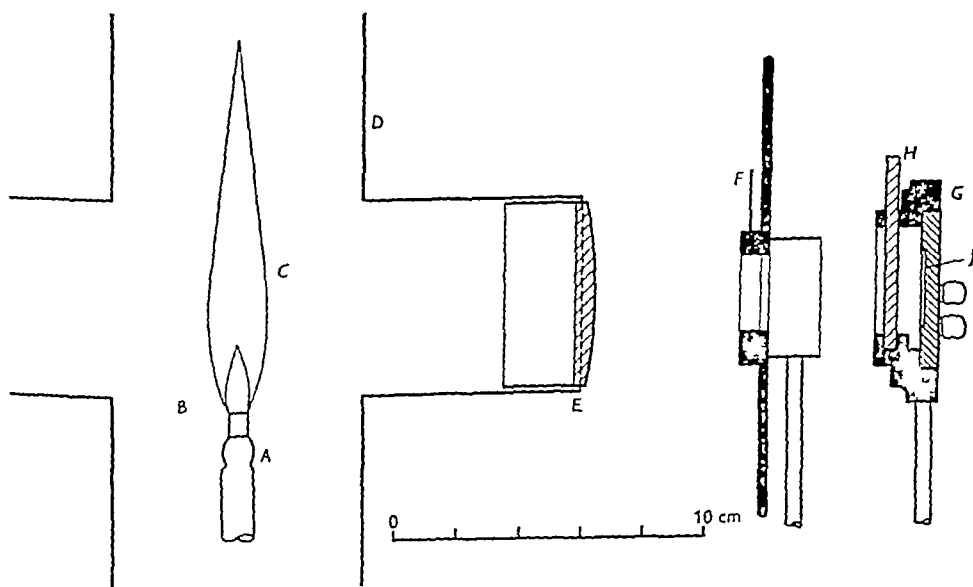


Fig 6. Optical system for determination of sodium. *A*, burner, *B*, inner cone, *C*, outer cone of flame, *D*, chimney, *E*, plano convex lens (focal length 9 cm.), *F*, iris diaphragm (maximum aperture, 25 mm, mounted in shield), *G*, holder for optical filters *H* and selenium cell *J*

Determination of potassium The burner and chimney are arranged as for the determination of Na, except that the plano convex lens is in the left-hand end of the chimney. An iris diaphragm is mounted 15.5 cm from the centre line of the burner, and a photocell and filter (Fig. 7) are mounted with the centre line of the cell 25 cm from that of the burner. A gas filled caesium cathode cell (Type GS18, Cinema Television Ltd., Crystal Palace, London, S.E.) and an Ilford spectrum deep red filter (no. 609) are suitable for the

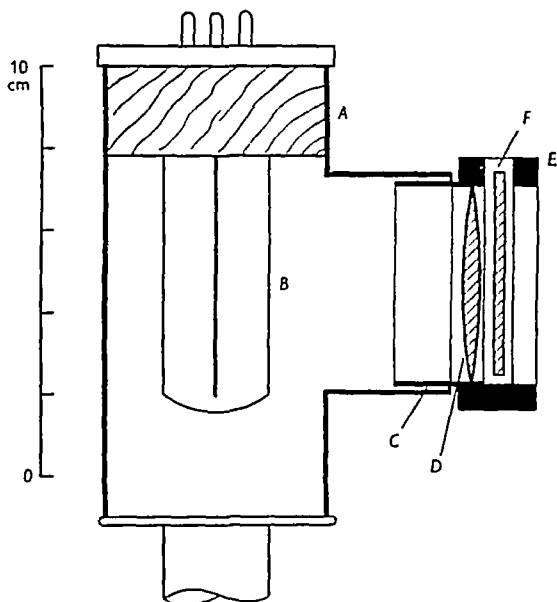


Fig. 7 Photocell and filter for determination of potassium. A, holder for photocell B, C, tube sliding into the front aperture of A carrying a biconvex lens D (focal length 6 cm) and a holder E which supports the optical filter F.

measurement of the 766 and 770 m μ K lines. A potential of 72 V is applied to the cell from a dry battery, and the photoelectric current is measured with the same galvanometer as for Na. A resistor and capacitor in parallel (1 M Ω , 0.5 μ F, time constant about 1 sec) are placed in the h.t. lead from the battery to damp variations in h.t. current.

OPERATION

The instrument is assembled without its chimney, and the air is then turned on and adjusted to 40 cm pressure. Water is placed under the inlet tube and this tube, the air inlet and the baffle plate are adjusted until a maximum spray is produced. The acetylene is then turned on, and the burner is lit. The flame should resemble a non-luminous Bunsen flame and should burn quietly. If flashes of white light appear in the flame, more air is needed. If the flame burns noisily, less air is needed. The behaviour of each burner must be studied individually, careful adjustment of the flame is necessary, since an unsatisfactory flame gives very erratic results.

The chimney is then put in place, and the appropriate photocell connected to the galvanometer. Further adjustment of gas and air pressures may be desirable to steady the flame so as to obtain a steady galvanometer reading.

A standard solution (NaCl or KCl, containing 2–4 mg Na or K/100 ml is suitable) is then fed into the atomizer, and the latter is adjusted until the galvanometer gives a maximum reading. The standard solution is replaced by water and the galvanometer is adjusted to read 0. The instrument is now ready for use.

If it were possible to keep the atomizer and burner working in a constant manner, it would be sufficient to calibrate the instrument for each run by passing a series of standard solutions through it (e.g. NaCl solutions containing 0.5, 1.0, 1.5, etc. mg Na/100 ml) at the start of the run, and drawing a calibration curve. However, since conditions are liable to vary, we find it best not to rely on a calibration curve, instead, after spraying each sample for analysis, we

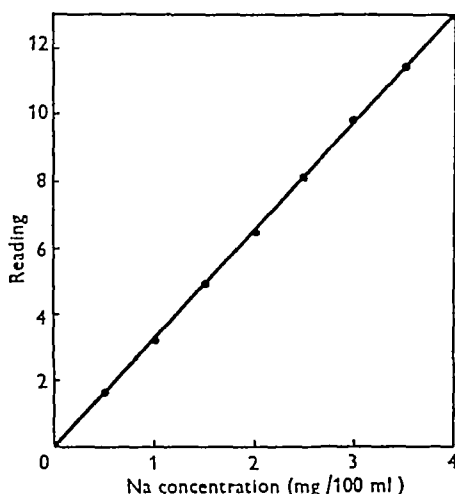


Fig. 8 Flame photometer readings for sodium. Standard curve obtained with pure NaCl solutions. (All readings in Figs. 8–13 are galvanometer readings on the reciprocal logarithmic scale described on p. 402.)

spray two standards, one of higher and one of lower concentration, and obtain the result by interpolation. The galvanometer readings on a logarithmic scale are directly proportional to the Na or K concentration, at least over short ranges (Figs. 8 and 13).

The instrument takes in solution at the rate of about 10 ml/min, and a sample with the two appropriate standards can be sprayed twice in 3 min. The instrument responds almost immediately to changes in the solution fed, and it is not necessary to wash through with water between each pair of solutions.

When a series of samples has been sprayed, the instrument is cleaned by thorough spraying with water, and then ethanol is sprayed. While the ethanol is burning at the flame, the acetylene is turned off and then air is drawn through for about a minute. The use of ethanol prevents the flame from back firing when the acetylene is turned off.

RESULTS

Solutions of pure sodium and potassium salts

Over the ranges which are required for sodium and potassium determinations on body fluids (0–5 mg/100 ml) the relationship between galvano-

meter readings and sodium or potassium concentrations is either linear or so near to linear that simple interpolation between two standards is possible. Specimen results are shown in Figs 8 and 13.

Interference by anions In view of the results obtained by other workers on the effect of anions on flame photometer readings (Barnes *et al* 1945, Crismon, 1948, Shapiro & Hoagland, 1948) solutions

- (1) Potassium increases slightly the light emission of sodium (see Fig 10)
- (2) Sodium increases the light emission of potassium (see Figs 12 and 13). The effect is not due to the sodium *D* line passing the filter, since pure sodium chloride solutions give very small readings (Fig 12). This effect can be eliminated by the use of a butane air flame.

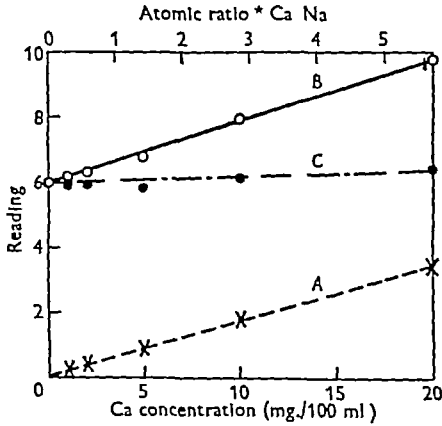


Fig 9 Flame photometer readings for sodium. Effect of calcium. *A*, readings for calcium (as CaCl_2), *B*, readings for calcium solutions, each containing 2 mg Na/100 ml, $C = B - A$ (* These values refer to curve *B* only)

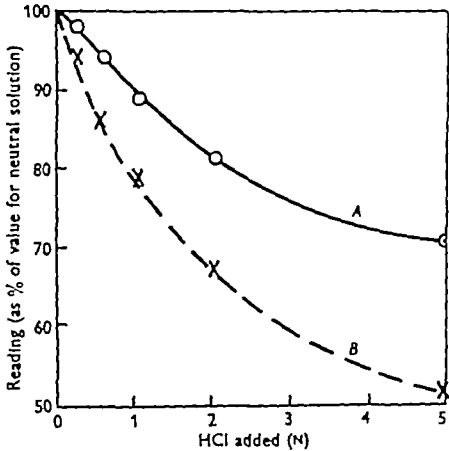


Fig 11 Flame photometer readings for sodium and potassium. Effect of acid. *A*, 2 mg Na/100 ml. (as NaCl) in all solutions, *B*, 2 mg K/100 ml. (as KCl) in all solutions, HCl added as shown by abscissae

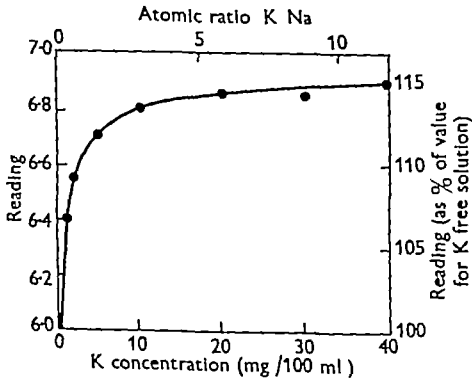


Fig 10 Flame photometer readings for sodium. Effect of added potassium, 2 mg Na/100 ml. (as NaCl) in all solutions, K (as KCl) added as shown by abscissae

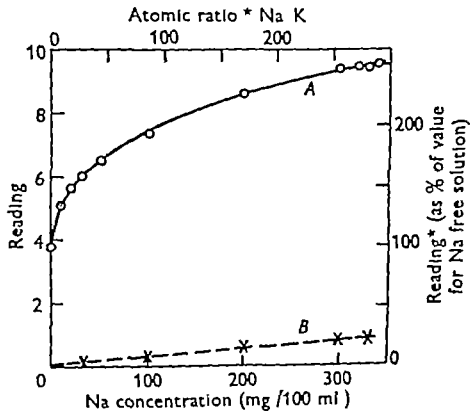


Fig 12 Flame photometer readings for potassium. Effect of sodium. *A*, solutions containing 2 mg K/100 ml (as KCl) + Na (as NaCl) as shown by abscissae, *B*, solutions containing no K, but Na as shown by abscissae (* These values refer to curve *A* only)

of the nitrates, sulphates, dihydrogen phosphates, monohydrogen phosphates, carbonates and acetates of sodium and potassium, each containing 3 mg Na or K/100 ml were sprayed. In all cases the galvanometer readings were identical with those for chloride solutions of the same sodium or potassium concentration.

Mutual interference of cations The following results have been obtained with the instrument described in this paper

- (3) Some light emitted by calcium ions (presumably the 618 and 620 $m\mu$ lines) passes the sodium filters. The intensity of this light is not sufficient to cause any significant difference in the sodium readings on plasma. Results for solutions containing sodium and calcium show that the

galvanometer readings are the sum of those for the corresponding concentrations of sodium and calcium in separate solutions (see Fig 9)

(4) No calcium light passes the potassium filter, and there is no interference between calcium and potassium in the flame

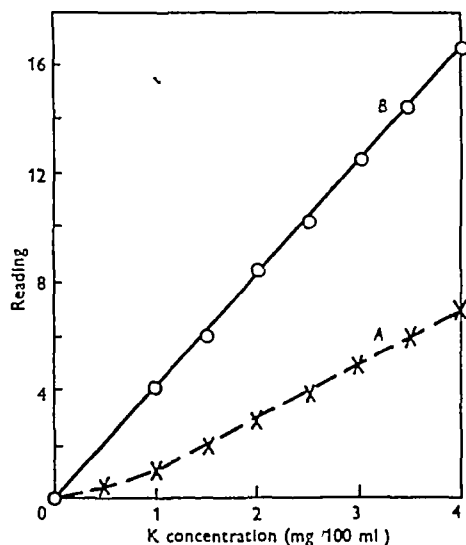


Fig 13 Flamephotometer readings for potassium Standard curves A, KCl solutions, B, KCl solutions each containing 330 mg Na/100 ml (as NaCl), used as standards in serum and urine K determinations

(5) The presence of acid lowers the galvanometer readings for sodium or potassium This lowering is not significant for acid concentrations below 0.2N (see Fig 11)

(6) Ammonia added as ammonium chloride (20 mg NH_3 /100 ml) to solutions had no effect on the photometer readings for sodium or potassium

Applications in medical biochemistry

The use of the flame photometer in the determination of plasma sodium, serum potassium, urine sodium and urine potassium has been discussed by Hald (1947), who compared the results obtained by the flame photometer and by chemical methods

Plasma sodium Plasma (0.2 ml) is washed from an Ostwald pipette into water (19.8 ml) giving a 1:100 dilution These solutions are then sprayed and compared with sodium chloride standards containing 2.5, 3.0 and 3.5 mg Na/100 ml It is not necessary to remove protein, and potassium and calcium (in the quantities present in plasma) do not interfere A specimen protocol showing duplicate readings for test and standard solutions is given in Table 1

The precision of the method was tested as follows One hundred samples of standard sodium chloride solutions, of concentrations from 250 to 350 mg Na/100 ml, representing plasma sodium values, were diluted and sprayed by the method described The root mean square difference between 'found' and 'calculated' values was 5.4 mg Na/100 ml Quality control limits for single observations in routine work are therefore as follows 90% limits, ± 8.9 mg Na/100 ml, 99% limits, ± 14.0 mg Na/100 ml

Table 1 Determination of sodium in plasma Specimen flame photometer readings and results

Sample	Plasma (diluted 1:100) Readings	Sodium standards (mg Na/100 ml)				Results (mg Na/100 ml)
		2.5	3.0	3.5	4.0	
A	6.0	—	5.5	6.4	—	328}
	6.1	—	5.6	6.4	—	330}
B	5.2	4.3	5.4	—	—	291}
	5.3	4.4	5.6	—	—	287}

Urine sodium These determinations are carried out as for plasma sodium determinations, 1:100 dilution is usually suitable

Serum potassium Normal serum contains about sixteen times as much sodium as potassium, and with this apparatus it is necessary to allow for this by adding sodium to the potassium standards used for comparison Fig 12 shows that for a set of sera (W, X, Y, Z) containing 20 mg K/100 ml and varying concentrations of sodium (say 250, 300, 330 and 350 mg/100 ml) the flame photometer readings for potassium after dilution 1:10 with water would show considerable differences These differences may be reduced to negligible proportions by diluting the sera not with water, but with a solution of sodium chloride containing 330 mg Na/100 ml (representing the mean of normal serum sodium values) If the above sera were diluted 1:10 with this solution, the resulting solutions would have the following concentrations (all in mg/100 ml)

	Original K	Original Na	Final K	Final Na
W	20	250	2.0	322
X	20	300	2.0	327
Y	20	330	2.0	330
Z	20	350	2.0	332

The galvanometer readings for 2.0 mg K/100 ml solutions containing 320–340 mg Na/100 ml are identical within the limits of experimental error Thus, if sera are diluted with this solution containing 330 mg Na/100 ml and used with potassium standards containing 330 mg Na/100 ml, the results

are dependent only on the potassium concentrations of the sera

A series of twenty two sera were analysed for potassium by this method and by the colorimetric method of Abul Fadl (1949) The results, with two exceptions, agreed within 2 mg K/100 ml and the root mean square deviation was 1.7 mg K/100 ml Potassium added to normal serum up to a total concentration of 40 mg K/100 ml could be determined with an accuracy of ± 1 mg K/100 ml

Urine potassium If urines are diluted with the 330 mg Na/100 ml solution and compared in the flame photometer with the potassium standards used for sera, the results are considerably higher than those obtained by the cobaltinitrite method of Abul Fadl (1949) However, if the urines are evaporated to dryness and ashed overnight at 400°, flame photometer determinations on the ashed material show tolerable agreement (within $\pm 10\%$) with the cobaltinitrite results (Table 2) Preliminary attempts to trace the cause of the discrepancies in the unashed urine have had no success The addition of urea (in the quantities commonly found in urine) to potassium standards did not affect the flame photometer results

Table 2 *Determination of potassium in urine*
Comparison of results by flame photometer and colorimetric methods

(Flame photometer results for unashed urine were obtained by diluting the fresh urine with 330 mg Na/100 ml solution Results for ashed urine were obtained by ashing duplicate samples by the procedure given on this page and carrying out flame photometer and colorimetric determinations (Abul Fadl, 1949) on the ash.)

Urine sample	Potassium concentrations (mg /100 ml)			Differences (%)	
	Unashed, flame photo- meter	Ashed, flame photo meter	Ashed, colori metric	$100 \frac{a-c}{c}$	$100 \frac{b-c}{c}$
	a	b	c		
A	92	77	80	+15	-3
		74	70	+37	+6
B	175	153	145	+21	+6
		153	155	+16	-1
C	166	150	155	+7	-3
		144	140	+18	+3
D	237	216	240	+1	-10
		222	245	+3	-9
E	230	197	200	+15	-2
		188	175	+31	+7
F	395	325	300	+31	+8
		380	340	+16	+12

The following method has been found suitable
Urine (2 ml) is evaporated to dryness in a porcelain crucible on the steam bath The residue is charred by

gentle heating with a Bunsen burner and ashed overnight in a muffle furnace at 400° The colourless ash is dissolved in 5 ml of a solution containing 5.43 g NaCl and 3.65 g HCl/l (330 mg Na/100 ml, 0.1N in HCl) and the resulting solution (1 or 2 ml, according to K concentration of urine) is diluted to 20 ml with NaCl solution containing 330 mg Na/100 ml, giving a final dilution of 1/50 or 1/25 with respect to the original urine The solutions are then compared with potassium standards containing 330 mg Na/100 ml

DISCUSSION

The literature contains a number of references to the mutual interference of metals in flame photometry The most thorough survey is that of Parks, Johnson & Lykken (1948), who found that in the Perkin-Elmer Model 18 photometer, burning natural gas, sodium readings were lowered by the presence of many metallic salts, including potassium salts, and also by ammonium salts and by acids, potassium readings were lowered by the presence of many metallic salts, including sodium salts, and also by ammonium salts and by acids Berry, Chappell & Barnes (1946) obtained similar results using an instrument built by the American Cyanamid Co

Several continental workers (Jansen, Heyes & Richter, 1934, Rauterberg & Knippenberg, 1940, Riehm, 1945, 1948, Boon, 1945) using instruments burning acetylene, which gives a higher flame temperature than the natural gas used in the American instruments, have found, like the present authors, that the foreign metals increase the readings for sodium or potassium In some cases (Jansen *et al* 1934, Boon, 1945) the interference effects depended to some extent on the type of optical system used (glass filter, Christiansen filter or monochromator), and some or all of the increase in the readings may have been due to the fact that the optical systems were not isolating the required spectral lines Riehm (1948) states that sodium increases potassium readings and that potassium increases sodium readings when acetylene is burnt, if, however, illuminating gas is burnt in the same apparatus these two metals do not interfere The Perkin Elmer Corporation (1945) also state that with their Model 18 photometer, lithium, sodium and potassium interfere mutually when acetylene is used, but not when illuminating gas is used

So far as the effects of anions present in the solutions are concerned, Parks *et al* (1948) and Berry *et al* (1946) found that the nature of the acids added to solutions of sodium or potassium salts influenced the results considerably Crismon (1948) found that solutions containing phosphate gave low results for sodium and potassium Shapiro & Hoagland (1948) found that phosphate had no influence on potassium

results, they pointed out, however, that they were using much lower concentrations than Crismon

It appears from these scattered and rather confused data that an investigation of these interference effects from the standpoint of the spectroscopist would be desirable

SUMMARY

1 The construction and operation of a photo electric flame photometer are described

2 The use of the photometer for the determination of sodium and potassium in blood and urine is described

3 The interference between various cations in the flame is discussed with reference to previous work on the subject

We are indebted to Prof E J King for his interest and encouragement, to Messrs C Lordan and W Weedon for constructing the photometer in use at the Postgraduate Medical School, to Messrs R A Brennan and J Gray for much technical assistance, and to Miss S Tompkins for drawing the figures

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Vitamin A in Seals

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(Received 22 February 1949)

Specimens of livers of polar bears and bearded seals (*Erignathus barbatus*) collected during the 1939-40 expedition to north east Greenland (Rodahl, 1943) were found by Rodahl & Moore (1943) to be very rich in vitamin A. During a further expedition to the sealing grounds off Newfoundland and Labrador, carried out under the auspices of the Royal Norwegian Government between March and May 1941, the vitamin A content of seal liver was further investigated. During this expedition the livers of hooded seals (*Cystophora cristata*) and Greenland seals (*Phoca groenlandica*) were weighed and assayed for vitamin A (considerable work on the extraction of vitamin A from this source was also carried out), whilst a limited

number of determinations was made on other tissues. The present paper deals also with the results of similar analyses by one of us of material from a single specimen of the Atlantic seal (*Halichoerus grypus*), obtained off the Pembrokeshire coast in October 1946, from two specimens of the common seal (*Phoca vitulina*) obtained in the Wash in August, 1947, and from the four specimens of *Cystophora cristata* collected in the pack-ice east of Greenland in July 1948.

EXPERIMENTAL

Vitamin A was determined colorimetrically, following the alkaline digestion procedure described by Davies (1933), except for the four 1948 specimens in which the determinations were made spectrographically. All specimens from the sealing grounds off Newfoundland and Labrador were dealt

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with in a laboratory on board ship, while those from Pem brokeshire and the Wash were transported to Aberystwyth in large vacuum walled flasks packed with solid carbon dioxide

Except in the case of baby seals, the stomachs of all specimens of the four species examined were empty of foodstuffs

Hooded seals (Cystophora cristata)

A total of sixty livers of this species was examined during March and April 1941. The results are summarized in Table 1

Table 1 *Livers of hooded seals*

(a) Liver weights (g)				
Sex and age	Mean	Range		
Baby seals, about 7 days old	1494	1000-2100		
Females, fully developed	2929	2200-3750		
Males, fully developed	4136	3600-5000		
(b) Mean liver vitamin A reserves (i u / g)				
Specimen	No of animals	March 1941	April 1941	Combined samples
Baby seals				
Foetus	1	120	—	—
1 hr after birth	1	150	—	—
7 days (approx)	1	300	—	—
14 days (approx)	4	1500	—	—
21 days (approx)	9	—	300	—
Young seals				
1 year (approx)	2	—	—	2400
2 years (approx)	2	—	—	1200
Fully developed				
Males above 4 years	26	5180	4320	4934
Females above 4 years	14	1370	2742	2110

Of the total of twenty six adult male hooded seals, eighteen were examined in March and eight in April, while a total of seven adult females were examined in March, and the same number in April

RESULTS

There is some evidence to indicate that the vitamin A reserve varies at the different breeding grounds. A further investigation of more material should therefore be carried out at other breeding grounds and at other periods of the year.

In this connexion it may be of interest to note that spectrographical examination of samples of liver from four hooded seals caught in the pack ice east of Greenland, latitude 74° N, in July 1948, showed lower vitamin A reserves than in the same species caught at the Newfoundland sealing grounds. The results are given in Table 2.

The oil content of the livers of hooded seals up to the age of 3 weeks is usually about 9%. After this age the oil content remains fairly constant at approximately 2.4%. The results of the vitamin A analyses of the liver oil of this species have been combined with the essentially similar results for Greenland seals, and are summarized in Table 6.

Table 2 *Vitamin A content of livers of hooded seals caught in the pack ice east of Greenland, July 1948*

Sex	Age	Fat in liver (%)	Vitamin A in liver (i.u./g)
Male	4 months	5.2	450
Female	4 months	3.6	350
Male	16 months	4.3	300
Male	4 years	4.1	450

Greenland seals (Phoca groenlandica)

A total of 145 livers of Greenland seals was examined between March and May 1941. The combined results are shown in Table 3.

Table 3 *Livers of Greenland seals*

(a) Liver weights (g)			
Sex and age	No of animals	Mean	Range
Baby seals			
Newborn	1	361	—
8-10 days	1	814	—
Young seals			
Males, 1 year	10	1142	769-1600
Females, 1 year	14	1050	900-1200
Fully developed seals			
Males	17	1783	1600-2250
Females	28	1681	850-2180

(b) Mean liver vitamin A contents (i u / g)

Sex and age	No of animals	Mean	Range
Baby seals			
Foetus	1	240	—
8-10 days	7	340	120-600
28 days approx	10	3000	—
Young seals			
Males, 1 year	24	1620	600-4500
Females, 1 year	23	1580	600-1800
Females, 3 years	10	5000	800-7500
Fully developed seals			
Males above 4 years	47	6570	3000-12000
Females above 4 years	23	2542	600-4500

With some livers separate determinations were made on portions from the right and left quadrate and caudate lobes, but in all instances an even distribution throughout the liver was found.

The possibility of a correlation between liver vitamin A reserve and physiological condition at one time of the year was tested on a series of thirty six animals caught on 19 and 26 April 1941. In each case a record was made of the approximate age, sex, thickness of subcutaneous fat (blubber) and any abnormalities. In all cases it was noted that the stomach was empty. The results in Table 4 show that no obvious correlation emerged.

Table 4 *Vitamin A content of livers from Greenland seals with reference to physiological conditions*

Sex	Approx age (years)	Thickness of blubber (cm)	Liver wt (kg)	Oil (%)	Vitamin A content		
					Liver (i u /g)	Oil (i u /g)	Total in whole liver (i u × 10 ⁻⁶)
					10 April 1941		
Male	>4	2.5	1.0	3.0	750	25,000	1 425
Male	>4	3.5	1.0	2.0	3,000	150,000	5 700
Male	>4	3.3	1.8	2.8	3,000	101,300	5 400
Male	>4	2.3	1.8	2.8	1,800	64,300	3 240
Male	>4	3.5	—	2.4	750	31,250	—
Male	>4	3.4	—	2.0	300	15,000	—
Male	>4	2.3	—	2.0	1,500	75,000	—
Male	>4	3.5	2.1	5.0	12,000	240,000	25 200
Male	4	3.8	2.25	5.0	15,000	300,000	33 750
Male	4	3.5	1.5	4.0	240	0,000	0 360
Male	4	3.5	2.25	3.6	1,200	33,500	2 700
Female	4	4.2	0.85	4.0	2,400	60,000	2 040
Female	4	4.3	1.85	3.0	1,500	50,000	2 775
Female	4	3.8	—	3.0	900	30,000	—
Male	1	1.8	1.25	4.4	900	20,450	1 125
Male	1	3.1	1.25	2.4	1,200	50 000	1 500
Male	1	2.1	1.6	3.0	240	8,000	0 384
Male	1	2.5	—	4.0	750	18,750	—
Female	1	2.5	1.0	2.0	1,500	75,000	1 500
26 April 1941							
Male	5	3.2	1.75	2.0	1 500	75,000	2 625
Male	5	3.3	1.0	2.0	12,000	600,000	22 800
Male	5	4.3	1.75	3.0	1,500	60,000	2 625
Male	5	4.0	1.7	4.0	2,400	60,000	4 080
Male	5	2.8	1.6	2.0	1,200	60,000	1 920
Female	5	3.5	1.75	3.0	3,000	100,000	5 250
Female	5	3.8	1.65	2.0	750	37,500	1 237
Female	5	4.5	1.5	3.0	1,200	40,000	1 800
Female	5	3.0	2.0	4.0	1,200	38,000	2 400
Female	5	4.1	2.0	4.4	2,400	54,545	4 800
Male	2	3.0	1.25	3.6	300	8,333	0 375
Male	2	2.8	1.35	3.0	1,200	40,000	1 620
Male	3	4.8	1.45	3.6	900	25,000	1 305
Female	1	2.8	1.0	3.6	2,400	66,666	2 400
Female	1	2.3	1.35	3.0	1,500	50,000	2 025
Male	1	4.4	1.1	3.0	3,000	100,000	3 300
Male	1	3.0	1.2	2.0	600	30,000	0 720
Mean results							
Male	5	3.2	1.82	2.7	3,208	151,588	7 975
Male	4	3.3	2.0	4.2	5,480	113,166	12 147
Male	1	2.4	1.09	3.4	1,165	39,023	1 487
Female	5	3.8	1.78	3.3	1,710	52,300	3 098
Female	4	4.1	1.35	3.3	1,600	55,000	2 408
Female	1	3.3	1.1	2.3	1,700	68,333	1 840

The fat contents of the livers showed a distribution in relation to age essentially similar to that of hooded seals, and are summarized in Table 5

Table 5 *Age and sex in relation to the fat content of the liver of Greenland seals*

Sex	Age	Mean fat (%)	Range (%)
Female	4 years	3.2	3.0-4.5
Male	4 years	2.8	2.0-5.0
Male	2 years	3.6	—
Male	1 year	3.4	2.4-4.4
Male	1 year	2.3	2.0-3.0
Male	8-10 days	10.0	—

The results of vitamin A analyses on the liver fat of both species are combined in Table 6 which indicates that there was in most instances an inverse relationship between the amount of oil and its vitamin A potency

The distribution of vitamin A in the organs of a single specimen of Greenland seal was studied, with the results shown in Table 7. The vitamin A reserve in the liver of this individual was only moderate and below the mean value for comparable specimens. Thus only small amounts were detected in the retina, kidney, lung and subcutaneous fat. The pancreas gave a positive reaction, but the con-

centration of vitamin A was too low to permit an accurate determination. The figures are in agreement with unpublished findings on organs of bearded seals (*Erignathus barbatus*) collected during the 1939-40 expedition to north-east Greenland.

Table 6 *Fat content of Greenland seal and hooded seal livers in relation to vitamin A potency*

Group no	No of animals	Oil (g/100 g)	Mean vitamin A content	
			Liver (i.u./g)	Oil (i.u./g)
1	20	2.0-2.4	3,675	134,687
2	3	2.5-2.9	2,000	104,300
3	10	3.0-3.4	1,459	51,444
4	4	3.5-3.9	1,200	53,374
5	7	4.0-4.4	1,470	38,500
6	8	5.0-5.4	13,500	270,000
7	8	9.0-9.4	300	33,300

Table 7 *Distribution of vitamin A in organs of a normal Greenland seal killed 4 May 1941*

Organ	i.u./g
Liver	2400
Kidney	6
Lung	3
Spleen	0
Pancreas	Trace
Testes	0
Muscle	0
Cerebellum	0
Cerebrum	0
Heart	0
Thyroid	0
Epididymis	0
Subcutaneous fat	12
Lymph gland	0
Corpus vitreum	0
Retina	4

The vitamin A content of a sample of milk collected from a female with a baby in its second week of life was 10 i.u./g.

Atlantic (grey) seal (Halichoerus grypus)

A single specimen weighing 59.5 kg, and estimated to be 18 months old, was shot by a fisherman off the Pembrokeshire coast in October 1946. Blubber (mean thickness, 3.4 cm) from the same specimen

Table 8 *Distribution of vitamin A in organs of an unmated Atlantic seal killed October 1946*

Organ	i.u./g
Liver	1,550
Liver oil	38,750
Lung	2.5
Left kidney	14.5
Right kidney	17.0
Suprarenals	Nil
Spleen	0.45
Subcutaneous fat	3.58

The total vitamin A reserve of the liver was calculated to be 2.8×10^4 i.u.

was forwarded to Prof. T. P. Hilditch, Department of Industrial Chemistry, University of Liverpool, for a study of its composition (Hilditch & Pathak, 1947). The liver, which weighed 1.81 kg, contained 4% of fat. The vitamin A content of the different portions analysed is shown in Table 8.

Common seal (Phoca vitulina)

Vitamin A estimations were carried out on tissues of two of a total of four specimens recovered from the Wash on a field trip made in July 1947. These two animals were a very young female (estimated as being not more than 2-3 weeks of age, blubber thickness 2.5 cm), and an immature male (estimated as not more than 4-6 weeks of age). The results are shown in Table 9.

Table 9 *Distribution of vitamin A in organs of two young common seals killed in July 1947*

Organ	i.u./g	
	Very young female	Immature male
Liver	12.0	90.0
Liver oil (8%)	15.0	180.0
Lung	Nil	0.6
Kidney	Nil	0.9
Suprarenal	Nil	Nil
Spleen	Nil	Nil
Subcutaneous	Nil	Nil

SUMMARY

1 The weight, vitamin A content and fat content of the livers of sixty-four hooded seals (*Cystophora cristata*), 145 Greenland seals (*Phoca groenlandica*), one Atlantic seal (*Halichoerus grypus*), and two common seals (*Phoca vitulina*) were determined.

2 In *Cystophora cristata* and *Phoca groenlandica* from northern waters during the breeding season (March to late May) the concentration of vitamin A reached very high levels in mature specimens, up to 24,000 i.u./g (mean 3,004 i.u./g) in *Cystophora cristata*, and up to 15,000 i.u./g (mean 3,441 i.u./g) in *Phoca groenlandica*. The mean liver vitamin A content of four specimens of *Cystophora cristata* taken in the pack ice east of Greenland in July 1948, was only 388 i.u./g.

3 The liver vitamin A content of the single specimen of *Halichoerus grypus* recovered from British waters fell within the range of the northern series, but those from the two young specimens of *Phoca vitulina* from the Wash were of much lower order.

4 There did not seem to be any correlation among mature specimens of any species between age, sex or thickness of the blubber and liver vitamin A.

5 The liver fat content in all species appeared to be high (8-10%) in very young specimens, and between 2 and 4% in mature animals.

6 Only relatively small amounts of vitamin A were found in the other tissues examined

Our thanks are due to the Royal Norwegian Ministry of Commerce for financing the scientific expedition to the Newfoundland sealing grounds, and to the Dunn Nutritional

Laboratory, Medical Research Council and University of Cambridge, for leave of absence of one of us to participate in the expedition, to Dr L Harrison Matthews for his encouragement and valuable co operation in the collection of material, and to Prof A N Worden and Dr Thomas Moore for their interest in this work

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A Method for the Estimation of Micro Amounts of Amino Nitrogen and its Application to Paper Partition Chromatography

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(Received 11 April 1949)

Since the introduction of paper partition chromatography (Consden, Gordon & Martin, 1944) it has been possible to separate completely many amino acids and peptides from complex mixtures. This has focused attention on the need for quantitative methods for estimation of micro amounts of these substances after their separation on paper chromatograms. The method of Pope & Stevens (1939) has proved of great use in the estimation of amino nitrogen at macro and semi micro levels. Preliminary notes of its use in a modified form for determining micro amounts of amino acids separated by single dimensional paper chromatography have already been given (Woiwod, 1948a, b). The present paper gives fuller details of the method, together with some preliminary results.

In the method of Pope & Stevens (1939) amino and 'peptide amino' nitrogen are estimated by allowing a suspension of copper phosphate to react with the material being investigated at pH 9.3, and, after filtration, the copper in the soluble complexes formed is estimated iodometrically. The terms 'amino nitrogen' and 'peptide amino nitrogen' just mentioned are used in this paper in the following sense. The groupings with which the copper phosphate reacts in proteins and their breakdown products are the α amino (or imino) group of free amino acids and the free amino groups of peptides and proteins, i.e. any not involved in peptide linkage. The nitrogen of the reacting group in free amino acids is designated α amino nitrogen and the nitrogen of the free amino groups in peptides and proteins is designated here as 'peptide' amino nitrogen. Little is known about the reactions of copper with peptide

amino nitrogen. The limitation this imposes when interpreting results with partial digests of peptides and proteins is discussed later.

EXPERIMENTAL

The method of Pope & Stevens (1939) can be modified so that micro amounts of amino N can be estimated by using the diethyldithiocarbamate reaction (Callan & Henderson, 1929) to determine the Cu in the soluble copper complexes. The method consists of the following stages: (a) the amino acid or peptide is allowed to react with a suspension of copper phosphate and the excess of the latter is then filtered off, (b) the Cu in a measured sample of the filtrate is determined by means of sodium diethyldithiocarbamate, (c) the Cu so determined is related to amino or peptide N, or weight of amino acid or peptide, by means of a standard curve or use of a factor.

The ratio, F , of weight of α amino N to weight of Cu reacting has been determined for a number of amino acids by this method (Woiwod, 1948b). The copper phosphate suspension of Pope & Stevens (1939) was used in the preliminary work, but its use had several disadvantages at micro levels. These were: (a) a considerable deviation of the value of F from the theoretical value of 0.44 for complexes of the type A_2Cu was observed with most amino acids, and the ratio was not constant from acid to acid, (b) the relationship between α amino N and Cu was not linear, (c) the reagent did not keep well and needed to be freshly prepared for each set of determinations, (d) the reaction had a large temperature coefficient, and (e) the blank value given by the reagent was large.

By omitting the $Na_2B_4O_7$ and adding Na_2HPO_4 to the Cu reagent a linear relationship between amino nitrogen and copper over a considerable concentration range was obtained, and the value of F for many amino acids was much nearer the theoretical value of 0.44. Thus it was possible to use this

factor for converting copper to amino N in comparative work not requiring the highest accuracy, and with mixtures of amino acids such as complete hydrolysates of protein. The elimination of $\text{Na}_2\text{B}_4\text{O}_7$ also increased the stability of the reagent, which was then no longer affected by temperature, and blanks were low and consistent. It was, however, not possible to obtain the same value of F for all amino acids. Mixing the components of the new reagent at room temperature gave a copper phosphate suspension the pH of which slowly increased on standing. At the same time the factor F obtained with standard amino acid solutions slowly decreased, and with glycine most nearly approached the theoretical value of 0.44 when the reagent had stabilized. This stabilization was assisted by heating, refluxing the reagent for 1 hr and leaving to 'age' 24 hr before use gave a satisfactory final product. Such a reagent has been used over a period of 1-2 months with no apparent change in properties when tested with a standard glycine solution. For determining amounts of α amino N of the order of 1 mg/ml and determining Cu iodometrically the original reagent of Pope & Stevens (1939) was more satisfactory as it gave a greater uptake of Cu/mol. of amino N than the modified reagent.

METHOD

Apparatus All glassware must be thoroughly cleaned to remove traces of Cu and a routine of cleaning rigidly followed if blanks are to be minimal and consistent. After use all glassware should be washed first with soap and water, then rinsed and transferred to a Pyrex glass vessel to soak overnight in N HCl . After soaking, all tubes are tested with an acid solution of sodium diethyldithiocarbamate, rinsed with distilled water (less than $0.02 \mu\text{g Cu/ml}$) and dried, and funnels are rinsed with distilled water.

Distilled water All reagents are prepared with good grade distilled water (less than $0.02 \mu\text{g Cu/ml}$). Where glass distilled water is necessary its use is mentioned in the text. The following reagents are required.

Cupric chloride Dissolve 27.3 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (A.R.) in boiled distilled water and make up to 1 l.

Trisodium phosphate solution Dissolve 25.6 g Na_2HPO_4 (A.R.) in 500 ml boiled distilled water, add 180 ml N NaOH and make up to 1 l with boiled distilled water.

Dissodium hydrogen phosphate Dissolve 25.6 g Na_2HPO_4 (A.R.) in boiled distilled water and make up to 1 l.

Sodium diethyldithiocarbamate Dissolve 2.0 g in 100 ml distilled water and filter. This solution precipitates slowly on standing and is refiltered immediately before use.

Standard copper solution Dissolve 3.928 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (A.R.) in boiled glass distilled water, add 1 ml concentrated H_2SO_4 (A.R.) and make up to 1 l. This solution is diluted 1 in 10 with glass-distilled water for use. One ml of this diluted solution contains $100 \mu\text{g Cu}$.

Copper phosphate suspension The $\text{Na}_2(\text{PO}_4)_2$ solution (1 vol) is added to 1 vol of the CuCl_2 solution and well mixed. The Na_2HPO_4 solution (4 vol) is then added, mixed, and the mixture boiled under reflux for 1 hr. The resulting copper phosphate suspension is allowed to 'age' for 24 hr before use.

Preparation of standard copper curve

All water used in the preparation of the standard curve should be glass distilled. Volumes of the standard copper solution containing between 1 and $100 \mu\text{g Cu}$ are pipetted into 5 ml distilled water, then 0.1 ml sodium diethyldithio-

carbamate solution is added and the volume made up to 10 ml with glass distilled water. The yellow solution is shaken for 15 sec with 10 ml amyl alcohol (A.R.), centrifuged to clear the amyl alcohol layer and the optical density of this layer determined by means of a Spekker absorptiometer using blue glass filters no. 7 (maximal transmission at about $440 \text{ m}\mu$). The curve relating Cu to optical density is linear up to $6.0 \mu\text{g Cu/ml}$.

Preparation of standard amino acid or peptide curve

For the highest accuracy it is necessary to prepare a standard curve for each specific amino acid or peptide. These curves are reproducible and once prepared only need checking from time to time with a standard solution. Glass distilled water is used in the preparation of the standard curve. The standard amino acid or peptide solution is added by means of a Trevan microsyringe (Trevan, 1925) to 2.5 ml of the Na_2HPO_4 solution, the added volume being 0.1 ml or less. Not more than $50 \mu\text{g } \alpha$ amino N or $25 \mu\text{g}$ of peptide amino N should be added. After thorough mixing, 2.5 ml of the copper phosphate reagent are added, the mixture is well shaken, allowed to stand for 30 min and filtered through pleated papers (Whatman no. 42, 9 cm) which prevent the copper phosphate suspension 'creeping'. The filtrate (2 ml) is pipetted into 8 ml of glass distilled water in a centrifuge tube, 0.1 ml of the diethyldithiocarbamate solution is added, mixed and stood for 10 min to allow the reaction to proceed to completion. The solution is extracted with 10 ml amyl alcohol with shaking for at least 15 sec, followed by centrifugation to clear the amyl alcohol layer. The optical density of the yellow solution is determined and the values recorded as μg copper by means of the standard curve. A blank determination is carried out in triplicate at the same time, the procedure being identical except that an equal volume of glass-distilled water is added instead of the solution containing the amino N. The blank value is subtracted and the corrected Cu value obtained is multiplied by the dilution factor. Thus a standard curve is obtained covering the range $1-50 \mu\text{g}$ of α amino N or $1-25 \mu\text{g}$ peptide amino N/5 ml.

Estimation of amino nitrogen

The procedure is the same as for the preparation of the standard curve, the unknown solution being added in place of the standard amino acid solution. If it is not possible to keep the volume added to 0.1 ml, any convenient volume may be used, provided a standard curve is prepared using the same volume. Progressive dilution will lead, however, to a falling off in the copper uptake and a loss of sensitivity.

RESULTS

A preliminary survey of some 25 amino acids and peptides has shown that alanyl glycine, histidine, hydroxyproline, aspartic acid, serine, threonine, valine, leucine, isoleucine, glutamic acid, methionine, asparagine, glycine, tyrosine, arginine, phenylalanine, cystine, cysteine, tryptophan, glutamine, α alanine, proline, lysine and ornithine all react satisfactorily with the reagent (Woivod, 1943a, b). Table 1 gives a list of average F values obtained with

some further amino acids and peptides including 'pantonnine' (α amino $\beta\beta$ dimethyl- γ hydroxybutyric acid, Ackermann & Kirby, 1948, Holly, Barnes, Komuszy & Folkers, 1948) It was not possible to examine in detail the behaviour of all these substances, but readings were made at 10 and 30 μg levels of α amino nitrogen or 5 and 15 μg of peptide

Table 1 *Ratio (F) for some amino acids and peptides of weight of α amino or peptide amino nitrogen to weight of copper reacting*

Compound	Average F value
$\alpha\gamma$ Diaminobutyric acid	0.25
α Amino n butyric acid	0.43
α Amino $\beta\beta$ -dimethyl γ hydroxybutyric acid	0.46
Glycylglycine	0.22
Diglycylglycine	0.23
Triglycylglycine	0.24
γ Aminobutyric acid	No reaction
4 Aminobutane 1 carboxylic	No reaction
5 Aminopentane 1 carboxylic	No reaction

amino nitrogen, and an average value for the factor F obtained from the slope of the curve drawn through these values. In most instances this curve passed through the origin, indicating a linear relationship between copper and nitrogen at least up to 30 μg of α amino nitrogen. At micro levels of amino nitrogen those acids which normally form sparingly soluble copper complexes, such as cystine, methionine, leucine and phenylalanine, all react well, presumably because the solubility product of their copper salts is not exceeded.

A more detailed study has been made of glycine, valine and leucine. These give linear curves up to 30 μg of α amino nitrogen, but deviate from linearity between 30 and 50 μg α amino nitrogen. Excessive dilution of the sodium phosphate solution is inadvisable as the amount of copper reacting/mole of α amino nitrogen is affected by the strength of this solution. The effect is shown in Table 2, where

Table 2 *The effect of Na_2HPO_4 concentration on the amount of copper reacting with glycine and lysine*

Amino acid	Na_2HPO_4 (% saturation)	Copper uptake (μg Cu/2 ml)	F value
Glycine (10 μg α amino N/5 ml)	8	6.4	0.62
	32	7.9	0.51
	60	9.7	0.41
	100	11.4	0.35
Lysine (10 μg α amino N/5 ml.)	8	4.8	0.83
	32	5.7	0.70
	60	7.5	0.53
	100	8.8	0.45

increasing strengths of disodium hydrogen phosphate solution up to saturation give increasing copper uptake with standard solutions of glycine and lysine. Ten μg amounts of α amino nitrogen were used in

every case, the figures in the third column being the copper value in a 2 ml sample of the filtrate. The blank copper value also rises slowly with increasing phosphate concentration.

It seems unlikely that any adjustment in the composition of the reagent will give the theoretical value of 0.44 with all acids. The final composition chosen is one which gives this value with glycine. This compound was used for much of the exploratory work. Most of the amino acids tested give values which lie fairly close to this theoretical value, suggesting the formation of complexes of the type $A_2\text{Cu}$. A number of α amino acids, however, behave in exceptional ways. Lysine and ornithine both react less readily than other amino acids, giving an average F value of about 0.5, whereas histidine and $\alpha\gamma$ diaminobutyric acid have approximately the same F value as peptides (0.22), indicating the formation of a complex of the type ACu . Mono amino acids with amino groups at other than the α position show little or no reaction with copper phosphate. β Alanine reacts very slightly and γ aminobutyric, 4 aminobutane and 5 aminopentane 1 carboxylic acids do not react at all. This failure of amino acids with the amino group at other than the α position to form copper salts has enabled Dent (1948) to separate such acids from α amino acids by running the copper salts of the mixed acids on two dimensional paper chromatograms. A mono amino acid with the amino group at a position other than α will not be affected by copper phosphate treatment, whereas the α amino acids will fail to appear in their usual positions and run to one side of the paper chromatogram. All the peptides so far tested have given values of F very near that expected for the formation of a complex PCu .

Application to paper chromatography

The copper phosphate reagent reacts satisfactorily with amino acids separated by paper chromatography (Woiwod, 1948a, b) and has been used to determine the ratio of leucine to threonine in a sample of polymyxin A (Jones, 1948). The amino acid is first located on the chromatogram by inspection in ultraviolet light, this involves no destruction of the acids or peptides. The paper is dried for 3–4 hr at 80° (Woiwod, 1949), and then examined in the dark under illumination from an ultraviolet lamp. The amino acids and peptides appear as light blue fluorescent spots on a dark purple fluorescent background.

A square of filter paper (approx $1\frac{1}{2} \times 1\frac{1}{2}$ in) with the amino acid or peptide spot on it is cut into strips about $\frac{3}{4} \times \frac{1}{4}$ in. These are dropped as a bundle into a dry test tube $6 \times \frac{5}{8}$ in, 2.5 ml of Na_2HPO_4 solution is added and the tube left to stand 30 min with occasional swirling. Copper phosphate suspension (2.5 ml) is then added and the mixture, after standing a further 30 min, is filtered. The filtrate is treated as described for the determination of amino N. A blank square of paper of the same size is cut from the paper and carried through an identical series of operations. The Cu value obtained after correcting for the blank is converted

to N or weight of material by means of a standard curve. The blank consists mainly of soluble copper from the copper phosphate suspension and is normally about 10 μg copper/ml of filtrate. A square of Whatman (nos 1 or 4) paper $1\frac{1}{2} \times 1\frac{1}{2}$ in gives no readable copper colour when tested without the addition of copper phosphate.

Early work with glycine and valine using *n* butanol with ammonia as the solvent give recoveries of amino N between 90 and 95% after chromatography (Woiwod, 1948*a*). After further development of the copper reagent, recoveries from chromatograms were re-investigated. The solvent used was

fluorescence often extended right to the solvent front. It may therefore be that failure of the acids to reach equilibrium with the solvent in the moving phase is partly responsible for the losses.

The rather large losses of amino N on paper chromatograms, particularly with faster running amino acids, probably accounts for the larger quantity of amino N needed to obtain good two-dimensional paper chromatograms. The amino acids, besides being depleted during the run, are also dispersed over three to four times as great an area as compared with a single dimensional paper chromatogram. For

Table 3 Recovery of amino nitrogen from chromatograms of single amino-acids on Whatman no 4 paper

Solvent	Amino acid	Approx R_F value	Before running (μg Cu/2 ml)	Recovery (μg Cu/2 ml)	Standard deviation (μg Cu/2 ml.)	Recovery (%)
Single dimensional paper chromatograms run 16 hr						
<i>n</i> Butanol acetic acid	Glycine	0.2	18.6	17.7	0.3	95.1
	Valine	0.4	28.8	26.1	0.6	90.7
	Leucine	0.6	21.6	17.6	0.8	81.5
'Collidine'	Glycine	0.1	18.6	17.5	—	94.1
	Leucine	0.4	21.6	17.6	—	81.5
Two-dimensional paper chromatograms run 16 hr each way						
<i>n</i> Butanol acetic acid and phenol	Glycine	—	18.6	16.1	—	86.5
	Valine	—	28.8	20.6	—	71.6
	Leucine	—	21.6	13.8	—	63.9

n butanol acetic acid (Partridge, 1948) run on Whatman no 4 paper, this combination of solvent and paper being used for routine single dimensional chromatography in this laboratory. Glycine, valine and leucine were studied. Ten spots of each substance were run for 16 hr, the spots located by ultraviolet light and the amino N determined therein. The recoveries, based on the copper in a 2 ml. sample of filtrate, are shown in Table 3, together with the standard deviation from the mean of the ten results at each level. The results indicated that the percentage recovery decreased with increasing R_F values. 'Collidine' gave a similar result with five spots each of glycine and leucine.

If the loss of amino N increases with the distance the spots travel, then decreasing recoveries should be obtained when the same substance is run on the chromatogram for increasing times. This is the case with glycine (Fig 1). Samples containing 20 μg α amino N were run for various times on Whatman no 4 paper with *n* butanol acetic acid as solvent, the paper removed, dried and determinations of amino N made. There was a progressively poorer recovery with increase in the distance the amino acid had travelled from the starting line. Little work has yet been done with recoveries from two-dimensional paper chromatograms. It was thought, however, worth seeing whether the method could be applied to such paper chromatograms. A mixture of glycine, valine and leucine was run in duplicate first with *n* butanol acetic acid and then with phenol as solvents, in that order. After 16 hr in each direction on Whatman no 4 paper, the spots were located by ultraviolet light and the amino N determined. The recoveries are shown in Table 3. Considerably greater losses occurred with the two-dimensional paper chromatograms than in the single dimensional runs. The losses, again, were greatest with the faster running compounds. It was thought that adsorption was the most likely cause of the losses. It was noted, however, that with single dimensional paper chromatograms a 'tramline' of

this reason more satisfactory two-dimensional paper chromatograms are obtained when the starting spot is kept as small as possible, preferably 0.1 in in diameter, or less (Woiwod, 1949).

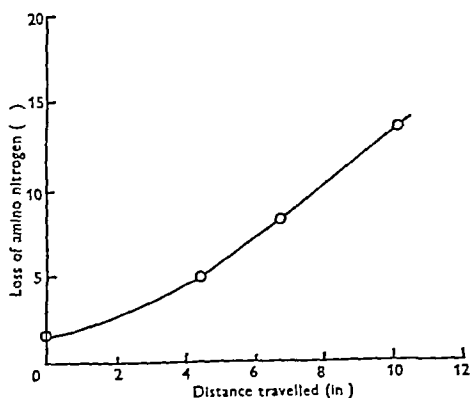


Fig 1 Curve illustrating the progressive loss of amino N with increasing distance travelled from starting line. Single-dimensional paper chromatogram run with *n* butanol acetic acid on no 4 Whatman paper. Original glycine concentration equivalent to 20 μg α amino N.

The destruction of amino-acids by ninhydrin on paper chromatograms

The usual procedure for locating amino acids on paper chromatograms is to spray with a solution of ninhydrin and then to heat. Coloured spots are obtained which indicate the position of the various amino acids and peptides. An investigation, by means

of the copper reagent, of the breakdown of amino acids occurring during ninhydrin colour development, indicated that considerable quantities of amino nitrogen remained after colour development was apparently complete. Before the copper reagent could be applied to ninhydrin sprayed papers, however, a method had to be found for blocking the reaction between excess ninhydrin on the paper and copper phosphate. The addition of 4.0 g/l of sodium borate to the disodium hydrogen phosphate solution completely inhibited the reaction. That there was not complete destruction of amino acids by ninhydrin was noted by Work (1948) who rechromatographed a spot already fully developed by ninhydrin and was able to obtain a coloured spot in a new position after respraying with more ninhydrin and heating. We have confirmed this observation. With glycine it has been found that after spraying with ninhydrin, heating for increasing times and estimating the residual glycine with copper phosphate reagent, there is a rapid initial destruction of amino nitrogen and then breakdown practically ceases. Respraying with ninhydrin solution and reheating causes little further destruction, nor is extra colour development obtained. This behaviour, illustrated in Fig. 2, would

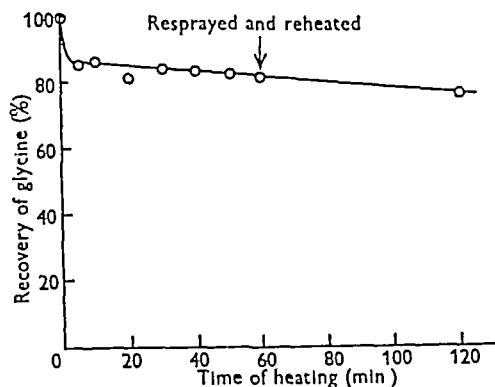


Fig. 2. Residual glycine, by copper phosphate estimation, on no. 4 Whatman paper after spraying with 0.1% ninhydrin in chloroform and heating. Original glycine concentration equivalent to 20 μ g α amino nitrogen.

be consistent with the formation of a substance inhibiting the reaction between ninhydrin and glycine on paper. The ninhydrin reaction is undoubtedly complex, as solutions of the coloured spots give a number of coloured bands on an alumina column, and it seems probable that among the products of the reaction such an inhibitor could be present. It was suggested (Woiwod, 1948a) that the failure of ninhydrin to destroy more than a portion of the amino acid in a spot on paper might enable acids to be located by this reagent before quantitative estimation. The difficulty in assessing the degree of

breakdown, however, led to abandonment of the method in favour of using ultraviolet light for locating the spots.

DISCUSSION

The copper phosphate reagent described here can be used to determine both the α amino group of amino acids, and the free amino groups in polypeptides and proteins which have been called 'peptide amino' groups in this paper. Although two conversion factors are therefore necessary, this raises no problems when determinations are made on solutions of isolated amino acids or peptides, as the necessary conversion can in most cases be achieved by means of standard curves prepared beforehand. With mixtures of α amino acids and peptides, however, such as those in partial hydrolysates of proteins, it is not possible to say with certainty what factor should be used for converting copper into amino nitrogen.

Pope & Stevens (1939) followed the digestion of fibrin by trypsin and showed that the results they obtained with their copper reagent agreed with those given by the gasometric Van Slyke method. A conversion factor which assumed that 1 mole of copper was equivalent to 2 moles of amino nitrogen was used. It may be that very long peptide chains reacted under their conditions in such a way that this factor was a fair approximation. However, the few peptides tested at micro levels (Table 1) react as if a complex PCu was formed. By the ninhydrin method of Van Slyke, MacFadyen & Hamilton (1941) it is possible to determine and to correct for free α amino nitrogen in a complex protein digest, and hence to obtain the peptide amino nitrogen, assuming a factor of 0.22 to hold in all circumstances when converting copper into peptide amino nitrogen. This assumption may not be justified in view of the lack of knowledge existing about the reaction of copper with peptide amino nitrogen. Though the ϵ amino group of lysine appears to be inactive in the free acid no information is available as to its reaction when the α amino group is in peptide linkage. Similarly, it is by no means certain how the imidazole group of histidine will react when its α amino group is in peptide linkage. In practice, however, these limitations are not found to detract from the usefulness of the copper reagent in following the rate of hydrolysis of a protein, when an arbitrary factor of 0.44 for converting copper to amino nitrogen is used. The presence of such acids as histidine or α -diaminobutyric acid in large amounts in peptides or proteins makes it difficult to assess the degree of breakdown of such materials, as both these amino acids form complexes of the type ACu , and amino nitrogen figures in excess of the total nitrogen figures can be obtained if the factor 0.44 be used. By plotting amino nitrogen against

time a guide to the completeness of the digestion can be obtained although the figures have no absolute significance. Attention is drawn to these points as ignorance of these effects might lead to faulty conclusions being drawn from experimental results.

The failure to obtain a theoretical conversion factor with many amino acids is at first sight puzzling, since it appears in some instances as if more copper were incorporated than the formation of the complex A_2Cu would allow. Borsook & Thumann (1932), however, showed that a number of copper complexes of both glycine and alanine can be obtained and can co-exist in solution, similar behaviour with other acids could account for the anomalous results obtained in the present work. Furthermore, it is not certain that the soluble copper salts estimated by the present method correspond to the isolated copper salts of the amino acids.

SUMMARY

1 A method is described for determining micro amounts of α amino nitrogen (1–50 μg) and peptide amino nitrogen (1–25 μg).

2 The method has been applied to single and two dimensional paper chromatograms of a number of α amino acids and an evaluation made of the losses involved during chromatography.

3 The destruction of amino acids on heating the spots on paper chromatograms developed with ninhydrin has also been studied quantitatively. It is suggested that a decomposition product makes the reaction self limiting.

I am indebted to Dr C E Dent for samples of the non- α amino acids investigated. I also wish to thank Mr R Knight for valuable technical assistance.

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Long-chain Unsaturated Fatty Acids as Essential Bacterial Growth Factors

SUBSTANCES ABLE TO REPLACE OLEIC ACID FOR THE GROWTH
OF *CORYNEBACTERIUM* 'Q' WITH A NOTE ON A POSSIBLE METHOD
FOR THEIR MICROBIOLOGICAL ASSAY

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(Received 19 April 1949)

Forty years ago, Fleming (1909) observed the stimulatory effect of oleic acid on the growth of *Corynebacterium acnes*, but it is only recently that the essential nature of long chain unsaturated fatty acids for the growth of certain organisms has been firmly established. It is now generally accepted that linoleic acid is essential for the proper nutrition of rats (see Hansen & Burr, 1946), while Fraenkel & Blewett (1947) have shown that it (or linolenic acid) is necessary for normal development of the moth *Ephestia kuehniella*. Benham (1941) has

reported that oleic acid is essential for the growth of the fungus, *Pityrosporum ovale*, while, amongst bacteria, oleic acid has been found necessary for the growth of some strains of *Corynebacterium diphtheriae* from a small inoculum (Cohen, Snyder & Mueller, 1941), for *Clostridium tetani* (Feeney, Mueller & Miller, 1943), for *Cl. sporogenes* in the absence of biotin (Shull, Thoma & Peterson, 1949), and for the unidentified *Micrococcus* 'C' (Dubos, 1947). There have also been reports, of which the most accurate and complete are those of Williams & Fieger (1946)

and Williams, Broquist & Snell (1947), proving the necessity of oleic acid or linoleic acid for some strains of lactobacilli, usually only in the absence of biotin

During experiments on *Haemophilus pertussis*, a chance contaminant was isolated which was found to have the property of symbiotically stimulating the growth of *H. pertussis* in nutrient broth and other media without blood (Pollock, 1948*a*, 1949). This organism proved, on further investigation, to be unable to grow in a medium of known composition without the addition of oleic acid. It was found to be an obligatory aerobic diphtheroid, fermenting glucose, but not lactose, sucrose, mannitol or dulcitol, and has been provisionally labelled *Corynebacterium* 'Q'. Its general properties and its clear cut response to oleic acid suggested that it might be a suitable test organism for a study of the function and metabolism of long chain fatty acids.

METHODS

Glassware. All glassware was thoroughly cleaned with hot chromic acid to remove traces of fat, and the 50 ml Erlenmeyer flasks used for growth experiments were plugged with special fine glass wool (Fibreglass Ltd.) previously extracted with methanol and acid cleaned (Pollock, 1948*b*).

Medium. 'Vitamin free' casein hydrolysate (Ashe), supplemented by tryptophan, cystine, salts and growth factors (pH 7.6) as follows. Basal medium (final concentrations): casein hydrolysate (Ashe, 'vitamin free'), 5.0, KH_2PO_4 , 4.5 mg/ml, NaOH, to pH 7.6, L-cystine, 0.0024, L-tryptophan, 0.0008, MgSO_4 , 0.02, FeSO_4 , 0.0015 mg/ml. Growth factors 'A': biotin 0.01, nicotinamide, 1.0, Ca pantothenate, 0.75, pyridoxine, 2.5, riboflavin, 1.2, aurocurin, HCl, 0.5, *p*-aminobenzoic acid, 0.45, haemin, 2.0 $\mu\text{g}/\text{ml}$. Growth factors 'B': guanine sulphate, 11.0, inositol, 9.0, uracil, 5.5, adenine sulphate, 10.0, cytosine, 5.5, pimelic acid, 0.8, folic acid, 1.0 $\mu\text{g}/\text{ml}$.

All tests classified in Table 1 were done in a medium consisting of casein hydrolysate, salts and growth factors 'A'. For the accurate curves depicting the relationship between growth opacity and fatty acid concentration (Fig. 1) both groups of factors 'A' and 'B' were included in the basal medium.

This was sterilized by Seitz filtration, the haemin was first autoclaved and added after the rest of the medium had been sterilized.

Inoculum. One drop of a 1/100 dilution of a standard suspension of cells grown on tryptic meat agar (without added oleic acid) for 24 hr and once washed with water. This was found to contain on an average 50,000 viable cells.

Incubation. Unless otherwise stated, cultures were incubated aerobically at 37°, and growth was recorded by eye after 1, 2 and 3 days as tr, tr+, +, ++, etc. Flasks were left for 7 days before recording a negative result.

Fatty acids. Trivial and systematic names of fatty acids mentioned in the text are as follows: brassidic, *trans*-heptacos-12-ene-1-carboxylic acid, dihydroxystearic, 8,9-dihydroxyheptadecane-1-carboxylic acid, dibromostearic, 8,9-dibromoheptadecane-1-carboxylic acid, erucic, *cis*-heptacos-12-ene-1-carboxylic acid, elacostearic, heptadeca-8,10,12-triene-1-carboxylic acid, elaidic, *trans*-heptadec-8-ene-1-

carboxylic acid, lauric, undecane-1-carboxylic acid, linoleic, heptadeca-8,11-diene-1-carboxylic acid, linolenic, heptadeca-8,11,14-triene-1-carboxylic acid, myristic, tridecane-1-carboxylic acid, oleic, *cis*-heptadec-8-ene-1-carboxylic acid, *aff* oleic, heptadec-1-ene-1-carboxylic acid, palmitic, pentadecane-1-carboxylic acid, palmitoleic, pentadec-8-ene-1-carboxylic acid, petroselinic, heptadec-5-ene-1-carboxylic acid, ricinoleic, 11-hydroxyheptadec-8-ene-1-carboxylic acid, stearic, heptadecane-1-carboxylic acid.

The fatty acids used were the purest that could be obtained. Palmitic and stearic acids and the first samples of linoleic and linolenic acids were gifts from Dr W. T. J. Morgan, lauric, myristic, petroselinic, elacostearic and the second samples of linoleic and linolenic acids were supplied by Prof. T. P. Hilditch, the four latter as methyl esters. These esters were hydrolysed by heating with NaOH for 30 min at 100° under N_2 . Pure oleic acid was obtained by distilling a commercial sample (Hopkin and Williams 'Purified' grade), followed by purification through dibromostearic acid (Holde & Gorgas, 1926) and redistillation *in vacuo*. Methyl palmitate was isolated from distilled sperm oil fatty acids. The methyl esters of the liquid acids prepared by the Pb salt method (Hilditch, 1940) were fractionated *in vacuo* using an 8 in. Fensko column fitted with a heated jacket and reflux ratio head. The fraction boiling at 138–139°/1.6 mm had an I_{D} number of 102 (Trappe, 1938), and gave an acid with an equivalent of 256. The dihydroxystearic acids were prepared by the methods of Wittecoff, Moe & Iwen (1948) and Scanlan & Swern (1940). Elaidic acid was prepared by the method of Bertram (1936). Tween 80 (Atlas Powder Co.) was purified according to Davis (1947). Ricinoleic acid and erucic acid were purified by partition chromatography (Howard & Martin, unpublished).

All fatty acids were dissolved in the minimum amount of NaOH needed to give a stable solution or emulsion and added to the basal medium without further treatment, apart from sterilization by autoclaving at 15 lb pressure for 20 min.

In experiments where growth was measured turbidimetrically, the Spekker absorptiometer was used, with neutral grey filter H 108, and the concentration of fatty acid added plotted against the opacity of the culture (expressed as $\log I_0/I$). The relationship between opacity and dry bacterial weight was established by making serial dilutions of a washed suspension containing a known weight of cells in the medium used for growth, and plotting a standard curve. The dry weight of organisms was measured by drying a suspension of cells twice washed with distilled water in an oven at 110° to constant weight.

RESULTS

Effects of different fatty acids

If strict precautions were taken to eliminate all traces of extraneous fat, no visible growth ever occurred in the basal medium. Attempts to simplify this medium were not very successful, although it was found that satisfactory growth occurred in a mixture containing eighteen amino acids, Fe^{++} , Mg^{++} , and eight 'A' growth factors (see 'Methods') together with 10 $\mu\text{g}/\text{ml}$ oleic acid. After this confirmation that 'Q' would grow as well in a medium of known composition as in the casein hydrolysate,

and since no growth occurred in the latter in the absence of oleic acid, it was decided to use the casein hydrolysate as a basis for subsequent tests rather than the amino acid mixture, for the sake of economy

Table 1 *Ability of different long-chain fatty acids to support the growth of Corynebacterium 'Q'*

(Medium casein hydrolysate + growth factors (see 'Methods'), incubation at 37°, aerobically, inoculum, about 50,000 cells)

Acid tested	Concentration ($\mu\text{g/ml}$)	Growth after		
		1 day	2 days	3 days
Oleic	25	tr	+	+++
	5	+	++	++
	1	tr +	tr +	+
Elaidic	25	+	++	+++
	5	+	+	++
	1	tr +	tr +	+
Petroselinic	25	tr	tr	+++
	5	-	tr +	+
	1	-	tr	tr +
Palmitoleic	25	-	-	-
	5	++	++	+++
	1	+	+	+
Linoleic	25	-	++	+++
	5	-	++	++
	1	tr +	+	+
Linolenic	25	-	-	-
	5	-	++	++
	1	tr +	+	+
Tween 80 (purified)	25	tr	++	+++
	5	tr	+	+
	1	-	-	tr
Lauroic	Brassicic	No growth up to 7 days, at conc of 1, 5, and 25 $\mu\text{g/ml}$		
Myristic	$\alpha\beta$ Oleic			
Palmitic	cisDihydroxystearic			
Stearic	transDihydroxystearic			
Ricinoleic	cisDibromostearic			
Erucic	transDibromostearic			

The results are summarized in Table 1. For oleic, elaidic, petroselinic, linolenic, linoleic and palmitoleic acids, the relationship between concentration of fatty acid in the medium and total yield of organisms in dry weight after incubation for 3 days is expressed in the form of curves based on accurate opacity measurements (Fig 1). None of the acids which failed to promote growth had any detectable inhibitory effect at a concentration of 25 $\mu\text{g/ml}$ in the presence of 10 $\mu\text{g/ml}$ oleic acid.

Three different preparations of oleic acid and two each of linoleic and linolenic acids gave substantially the same results. It will be noticed that the higher concentrations of palmitoleic, linoleic and linolenic acids were markedly inhibitory, and it was first supposed that this might be due to some toxic impurity present in relatively small amount, particularly, since the first samples tested of linoleic

and linolenic acids were nearly 20 years old. However, the second samples of these two acids, which were freshly prepared, kept as their methyl esters and tested on the same days as the hydrolysis to the free acid, gave exactly the same results as the original preparations. This 'double action' effect which characterizes the action of unsaturated fatty acids on a number of different bacterial species (see Pollock, 1949) is much more marked in the case of oleic acid acting on lactobacilli (Williams *et al* 1947).

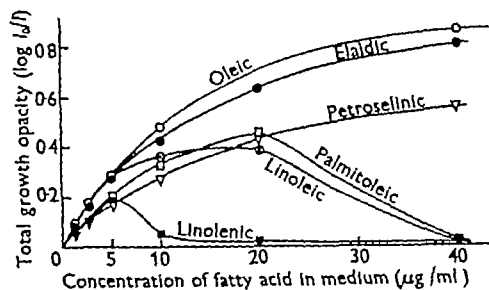


Fig 1 Relation between total final growth of *Corynebacterium 'Q'* and concentration of different fatty acids in the medium. Basal medium casein hydrolysate plus growth factors (see 'Methods'), incubation, aerobically at 37° for 3 days, inoculum, about 50,000 cells

It can be seen that the quantitative response to the active compounds is nearly the same, although petroselinic acid is not apparently quite so active as oleic acid, while with palmitoleic, linoleic and (especially) linolenic acid the picture is complicated by the superposition of an inhibitory effect on the growth promoting one. The most striking result is that shown by the 'unnatural' elaidic acid which gave almost exactly the same yield of cells as its isomer, oleic acid. However, with petroselinic acid it was observed that the rate of growth was considerably slower than with oleic acid (Table 1).

All saturated fatty acids tested were without effect. The action of purified Tween 80 (a polyoxyethylene derivative of sorbitan mono oleate) shows conclusively that the organism can utilize the oleate in this esterified form, the slight delay in growth compared to free oleic acid possibly being due to the need for preliminary hydrolysis, or to slower diffusion through the cell wall. Utilization of purified Tween 80 by certain oleic acid-requiring lactobacilli has also been reported by Williams *et al* (1947). Samples of ricinoleic acid and erucic acid gave, when first tested, slight growth in flasks containing the higher concentrations. This, however, was shown to be due to some residual impurity for, after passing these acids down a chromatographic column (Howard & Martin, unpublished), neither compound gave any growth whatever up to 25 $\mu\text{g/ml}$.

α -Elaeostearic acid was tested on several occasions, but the results were variable and have not been recorded. It was discovered that the iodine number of elaeostearic acid fell from 260 to 142 when the methyl ester was hydrolysed by heating 0.5 g at 100° with 2N NaOH, and continued to fall during incubation in M/30 phosphate buffer at pH 7.6 and 37°, reaching 32 in 5 days. Tests with this acid are therefore meaningless. This destruction of elaeostearic acid under aerobic conditions was also observed by Kodicek & Worden (1946).

Microbiological assay of oleic acid

The relative lack of specificity in the response of *Corynebacterium* 'Q' to fatty acids clearly reduces its value as an assay organism for oleic acid. Nevertheless, on occasions, the microbiological estimation of minute amounts of oleic acid (known to be the sole fatty acid present), or of oleic acid-like substances, has been of some value (see Pollock, 1949), particularly as there is yet no chemical method available. The basal medium employed was the same as that used in the ordinary growth experiments, with the addition of 'B' group of growth factors, and the technique of inoculation, incubation, etc. was similar. Satisfactory duplicates were obtained by incubating for 3 days in the usual way, but a linear response between concentration of fatty acid added and dry weight of cells produced was not obtained unless the flasks were shaken to promote maximal aeration. Table 2 gives the values obtained, with their standard deviations, up to a concentration of 10 μ g/ml of added oleic acid. It appears that concentrations of oleic acid between 2.0 and 10.0 μ g/ml could be assayed with an error of not more than 15%. Slight variations in the relationship between growth and concentration of oleic acid were found to occur from day to day. Flasks containing at least two concentrations of oleate were therefore set up and inoculated at the same time as the test, so that a fresh standard curve could be prepared each time. Tryptic meat broth and also 2% peptone water were found to be almost as satisfactory as the fat-free casein

hydrolysate medium for such estimations, although, of course, allowance has to be made for the slight growth (equivalent to an oleic acid content of about 0.4 μ g/ml in both cases) which occurs in the basal medium without added oleic acid.

Table 2 *Microbiological assay of oleic acid with Corynebacterium* 'Q'

(Flasks were incubated at 37° for 3 days on a mechanical shaker. Basal medium 'Vitamin free' casein hydrolysate with sixteen growth factors (see 'Methods'). Inoculum about 50,000 cells.)

Oleic acid added (μ g/ml)	No. of flasks	Mean yield of cells (μ g dry wt./ml)	Standard deviation
2.0	8	61	± 3.6 (6%)
5.0	8	144	± 4.2 (2.9%)
10.0	5	253	± 6.8 (2.7%)

A crude biological extract was prepared by heating a washed suspension of *Escherichia coli* for 30 min at 100° and spinning off the cell debris. The supernatant liquid was found to have a growth stimulating action for 'Q' corresponding to an oleic acid content of 41.5 μ g/ml, and the added oleic acid could be estimated satisfactorily in the presence of this extract diluted 1/9 (see Table 3). However, the assay of oleic acid in the presence of substances such as undenatured serum albumin or soluble starch, both of which have a high combining affinity for oleic acid, was found to be unsatisfactory. It is clear that an attempt to assay unsaturated fatty acids in biological material of unknown composition would have to be preceded by an extraction of all lipid with some fat solvent, or the demonstration that added oleic acid could be satisfactorily estimated in its presence.

DISCUSSION

In general, the results reported here for *Corynebacterium* 'Q' confirm and extend those of Williams & Fieger (1946) and Williams *et al.* (1947) with lactobacilli. The range of compounds able to replace oleic acid is relatively narrow. At least one double

Table 3 *Effect of crude biological extract on assay of oleic acid with Corynebacterium* 'Q'

(The crude extract used was an aqueous infusion of a boiled cell suspension of *Escherichia coli*.)

Extract present (ml)	Oleic acid added (μ g/ml)	Yield of cells (μ g dry wt./ml)	Total assay in terms of oleic acid (μ g/ml)	Oleic acid 'recovered'	
				(μ g/ml)	(%)
0	0	0	~	~	—
0	5.6	158	~	~	—
0	2.2	86	~	~	—
0.25	0	31	1.0	~	—
0.25	5.0	180	5.8	4.8	96
0.25	2.0	113	3.3	2.3	115
1.0	0	145	4.7	~	—
1.0	5.6	300	9.7	5.0	90
1.0	2.2	226	7.3	2.6	118

bond appears to be absolutely essential, but its exact position is less important. In the C_{18} series the 5,6 position* (petroselinic acid) is nearly as satisfactory as the 8,9 (oleic acid), although $\alpha\beta$ oleic acid (1,2 position) is inactive. The configuration around the double bond is apparently not critical, since elaidic acid has the same activity as its *cis* isomer, oleic acid. A single hydroxyl group, however, in the 11 position (ricinoleic acid), abolishes activity. Shortening the chain length by two methylene groups, leaving the double bond in the same position relative to the carboxyl group (palmitoleic acid), does not greatly reduce the activity, but the C_{22} acids, erucic and brassidic, with the double bond in the 12,13 position, are quite inactive.

The same relative lack of importance of the position of the essential double bond has been reported by Shull *et al.* (1949), who found that *cis* vaccenic acid ($\Delta^{10,11}$ isomer of oleic) would completely replace oleic acid for the growth of *Cl. sporogenes* in the absence of biotin. These workers also found that the *trans* isomers of both oleic and vaccenic acids were active, only less so than oleic acid itself. For lactobacilli, too, elaidic acid has been reported (Williams & Fieger, 1946) to be able to replace oleic acid.

The linear relationship between growth and fatty acid concentration of the medium suggests that oleic acid and its analogues are used to form some essential constituent of the cell protoplasm. The figures already quoted show that oleic acid consistently supports the growth of almost thirty times its own weight of cells of *Corynebacterium* 'Q'. Nevertheless, it must be emphasized that knowledge of the functions and metabolism of fatty acids in bacteria is scanty. The possibility that the growth promoting effect of oleic acid may be due to some physicochemical effect (e.g. on the permeability of the cell wall, as suggested by Kodicek, 1949), or purely to its acting as a specific source of energy, cannot be completely ruled out. It is also conceivable, although very unlikely, that the difference in growth promoting effect between active and inactive fatty acids is due possibly to very great differences in the ease with which they

* In this discussion the numeration is on the basis of Chemical Society nomenclature, in which $-\text{COOH}$ is a substituent on $C_{(1)}$.

may be absorbed through the cell wall. Moreover, the constancy of cell composition in relation to the quantity and quality of the nutrients provided is not, in general, so marked in the case of lipids as it is with protein and carbohydrate.

It is thus not easy to speculate on such questions as, for instance, whether elaidic acid is absorbed and utilized as such, or is transformed into oleic acid. The latter would appear more likely since elaidic acid has not yet been found to occur naturally in any organism. Sinclair (1935), however, has shown that it can replace up to 30% of the natural fatty acids in the phospholipins of liver and muscle if fed to rats, and Paul & McCay (1942) claim that guinea pigs will absorb (and probably 'utilize') 95% of the elaidic acid fed in the diet.

Clearly, such questions will not be finally answered until it is possible to investigate how far and in what way the fatty acid in the growth medium affects the fatty acid composition of the cells when grown. It is hoped to carry out further research along these lines, as well as to extend the range of compounds tested for their ability to replace oleic acid for growth of this organism.

SUMMARY

1. A number of long chain fatty acids have been tested for their ability to support the growth of an oleic acid requiring diphtheroid bacterium (*Corynebacterium* 'Q') in a medium of known composition.

2. None of the saturated fatty acids tested (lauric, myristic, palmitic, stearic) was active. Of the unsaturated acids, linoleic, linolenic, palmitoleic, elaidic and petroselinic acids were roughly equivalent to oleic acid, although the first three were inhibitory in the higher concentrations. Erucic, brassidic, $\alpha\beta$ -oleic and ricinoleic acids, however, were all quite inactive.

3. A possible method for the microbiological assay of oleic acid in concentrations between 2.0 and 10.0 $\mu\text{g/ml}$ has been outlined.

Our thanks are due to Prof. T. P. Hilditch, F.R.S., Dr W. T. J. Morgan, F.R.S., Dr J. H. Schulman and Dr R. E. Bowman for generous gifts of purified fatty acids, and to Dr R. Thomas of Unilevers for a gift of mixed sperm oil fatty acids. We also express our appreciation to Sir Paul Fildes, F.R.S., for his interest and encouragement.

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Phospholipin Metabolism in Rabbit-liver Cytoplasm

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(Received 14 April 1949)

The recent work of Fishler, Entenman, Montgomery & Chaikoff (1943) and of Entenman, Chaikoff & Zilvermit (1946) demonstrated that liver is the principal tissue in the body concerned with the production and removal of plasma phospholipins. However, it is not known whether synthesis and decomposition of phospholipins take place uniformly throughout the liver cell or whether these functions are associated with a particular type of particle in the cell. The present work deals partly with the technique of liver cell fractionation and partly with the study of the rates of renewal of phospholipins in liver cell fractions. Hevesy (1945) showed that the rate of renewal of phospholipin phosphorus in liver nuclei is slower than in the cytoplasm. His observation is confirmed, and it is shown that there are at least three different rates of renewal in different fractions of the liver cytoplasm.

1 ANALYSIS OF LIVER FRACTIONS

There have been numerous attempts to fractionate liver into components which can be directly related to visible granules in the liver cell. Several methods, e.g. those of Marshak (1941-2), Dounce (1943), Mirsky & Pollister (1946), have been used to prepare liver cell nuclei, while methods have been described by Claude (1946a), and more recently by Hogeboom, Schneider & Pallade (1948) for the fractionation of liver cytoplasm. Claude described the preparation of three fractions: (1) large granules, corresponding to the mitochondria and secretory granules of the histologist, (2) microsomes (small granules), composed of particulate elements of submicroscopic size, and (3) a supernatant, containing the particles and molecules which remain in solution after the first two fractions had been removed. Hogeboom *et al* (1948) have criticized Claude's (1946a) procedure on the ground that the granules isolated in fraction 1 are not identical morphologically or in staining

reactions with the mitochondria seen in the intact cell, and they have described a method of isolating these intact

MATERIAL AND METHODS

In an earlier (unpublished) series of experiments normal rabbit liver was fractionated by Claude's (1946a) procedure. The presence of glycogen, however, interfered seriously with the fractionation, as has also been observed by Claude. In subsequent experiments this effect was largely eliminated by withholding food from the animals the night before the removal of the liver.

In view of the evidence of Hogeboom *et al* (1948) that the large granules prepared by the use of a concentrated solution of sucrose more closely resemble the mitochondria of the intact cell, their method (in a slightly modified form) was used for most of the present investigation. In our work the first consideration has been the purity of the fractions, necessarily at the expense of information about the amount of each present.

Fully grown (2.3-2.8 kg) rabbits were used. They were anaesthetized with nembutal and ether, and after withdrawal of blood from the aorta, the liver was excised. All fractionation procedures were carried out at or near 0°.

Liver fractionation. Hogeboom *et al* used 0.88M sucrose solution in their procedure. For the sedimentation of the large and small granules in a reasonably short time from a solution of such high density, centrifugal forces of 24,000 and 41,000 g respectively are needed. A refrigerated centrifuge developing such forces was not available, and the following modifications were introduced to separate the particles at lower centrifugal forces.

The liver was pressed through a 1 mm mesh screen, 15-20 g of the resultant pulp were ground for 5 min in a mortar and 100 ml of 0.88M sucrose solution added over a period of 10 min with continuous grinding. The suspension was centrifuged at 600 g for 10 min and the deposit of intact liver cells, debris and nuclei discarded. The supernatant was centrifuged twice more at the same speed, the small sediments being discarded.

The suspension of cytoplasmic constituents, usually divided in two to four tubes, was now centrifuged at 18,000 g for 45 min to throw down the large granules. The supernatant, SII, was set aside. The deposits were pooled with the help of the small amounts of SII left behind and

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spun again to separate a little more SII. The residue of large granules was suspended in the sucrose solution and centrifuged at 18,000 *g* for 40 min, this was repeated three times, the supernatants being discarded. As previously observed by Hogeboom *et al.*, streaming birefringence could be readily demonstrated in the suspensions of large granules in sucrose solutions. After the purification, the preparation under the phase contrast microscope was seen to consist largely of rod like particles. For chemical analysis, the large granules were rapidly suspended in a saline bicarbonate solution (0.85% (w/v) NaCl containing 0.05M NaHCO₃), centrifuged at 18,000 *g* for 10 min and the deposit immediately dried from the frozen state.

The density of supernatant SII in four experiments was found to vary between 1.1091 and 1.1098 at 2°. This solution was dialysed under pressure against distilled water for 20–24 hr. The volume of the supernatant usually doubled during the dialysis, the density of the final solution varying between 1.019 and 1.011 at 2°. The dialysed supernatant, SII, was first clarified by centrifugation at 2000 *g* for 15 min, the small deposit discarded, and the clear supernatant from this centrifuged at 18,000 *g* for 2 hr. The sediment, a reddish brown transparent pellet, will be referred to as the small granule fraction. The supernatant from this was centrifuged at 18,000 *g* for 2 hr and the very small sediment which was usually deposited added to the first sediment. The final supernatant from this centrifugation, SIII, was dried from the frozen state.

The sedimented small granules were resuspended in the saline bicarbonate solution and centrifuged at 18,000 *g* for 2 hr. This was repeated, and the final deposit dried from the frozen state.

Chemical analysis. The purified granule fractions were analysed for total lipid, phospholipin, nucleic acid P and total N. Whole liver tissue (a dried sample of the liver pulp) was analysed for total lipid and phospholipin, and the supernatant fraction (SIII) for phospholipin.

The dried large and small granule fractions and the whole liver samples were extracted four times by boiling with an ethanol ether mixture (3:1, v/v) and finally once with ether. All the lipid was considered to be removed by this treatment. The fat-free residues of the large and small granule fractions were dried at 37°, the difference in dry weights before and after extraction was taken as the total lipid content of the sample, and was expressed as a percentage of the dry weight before extraction.

The combined extracts from each sample were evaporated to dryness under reduced pressure (20–40 mm Hg) with N₂ passing through the extract. The dry residue was extracted three times with light petroleum (b.p. 40–60°). Since the animals were injected with inorganic radioactive phosphate (see Part 2), it was necessary to get rid of traces of contaminating radioactive phosphate from the phospholipins. Ten drops of a saturated aqueous solution of Na₂HPO₄ were therefore added during the light petroleum extraction and the extracts vigorously shaken. It has been shown by Fries, Schachner & Chaikoff (1942) that shaking an ethereal solution of phospholipins with saturated Na₂HPO₄ solution effectively removed traces of radioactive inorganic PO₄³⁻ from the phospholipins. The extract was cleared by centrifugation, any particulate matter and inorganic phosphate being discarded in the lower aqueous phase. The clear extract was concentrated to about 2 ml, and the phospholipin precipitated by adding acetone and a saturated solution of ethanolic MgCl₂ as described by Bloor (1929). The phos-

pholipins were dissolved in moist ether, and phospholipin P estimated colorimetrically by Allen's (1940) method. Instead of HClO₄ as described by Allen, 5N H₂SO₄ and H₂O₂ ('100 vol') were used for the digestion. (The micro analytical reagent grade H₂O₂, supplied by British Drug Houses Ltd, was used throughout this work.) The dried supernatant SIII was similarly analysed.

The P and N contents of the fat-free, dry, liver fractions were determined on 10 mg samples in duplicate. The N content was determined by a micro Kjeldahl method, 50% (v/v) H₂SO₄ containing 1% (w/v) SeO₂ and H₂O₂ ('100 vol') were used for the digestion.

It was assumed that all low molecular weight P compounds were removed in the purification process and therefore the P remaining in the fat-free residue must be nucleic acid P. This P was multiplied by 1.7 to give nucleic acid N and by 9.01 to give the nucleic acid value. The nucleic acid N was subtracted from the total N to give protein N and this was multiplied by 6.25 to give total protein.

RESULTS

In Table 1 are given the values obtained by chemical analyses of whole liver and of the large and small granule fractions from it. The total lipid and more particularly the total phospholipin contents of whole liver are seen to be reasonably constant. In view of this, it is surprising that the total lipid of the large granule fractions is quite variable, which suggests that this fraction is not homogeneous. In appearance the large granule sediment was never homogeneous, two layers being always present. Attempts to separate these by further differential centrifugation, however, were not successful. The total lipid content of the small granules is greater and more constant than that of the large granules. This greater uniformity suggests that the fraction might be homogeneous. This was supported by preliminary electrophoretic experiments in a potassium phosphate buffer, of ionic strength *I* = 0.2 and pH = 8.0, in which a single boundary was apparent. The large granule fraction was not similarly examined.

It is suggested here that the centrifugal separation of the large from the small granules may depend mainly on the differences in their respective lipid contents, and hence on the differences in the density of the particles, rather than on the differences in their size.

The nucleic acid values of the large and small granule fractions show the greatest variation, the averages, however, being approximately the same in each. The variation may be due to the method of estimation, depending as it does on the assumption that all low molecular weight phosphorus compounds are removed in the purification process. Estimation of the ribonucleic acid content of these fractions by colorimetric methods based on ribose was unsatisfactory because of other interfering compounds, e.g. glucose from glycogen breakdown.

Table 1 *Lipid, nucleic acid and protein content of whole liver and of large and small granules obtained from it*

(All results are expressed as percentage of dry weight L = liver, LG = large granules, SG = small granules 'Total' (last two columns) = sum of total lipid, nucleic acid and protein)

Exp no	Total lipid			Phospholipin			Nucleic acid		Protein		Total	
	L	LG	SG	L	LG	SG	LG	SG	LG	SG	LG	SG
20	15.8	14.1	42.3	12.6	13.1	31.0	—	3.33	—	45.6	—	91.2
21	16.5	30.6	41.9	13.5	17.5	31.8	2.34	3.00	63.8	50.5	96.7	95.4
22	15.6	27.5	45.2	13.6	18.5	34.0	2.95	2.38	62.0	40.4	92.5	97.0
23	21.4	36.0	44.7	12.0	13.7	26.2	3.92	3.24	49.0	51.0	88.9	99.0
26	—	29.6	44.2	—	19.6	35.7	2.45	2.9	61.6	50.8	93.7	97.9
27	—	28.0	41.6	—	19.0	34.3	2.45	2.81	64.3	54.0	94.8	98.5
29	17.5	41.0	45.0	—	17.8	—	3.0	2.6	49.6	49.7	94.0	97.3
30	18.2	37.2	43.0	14.0	17.2	—	3.4	1.25	52.3	53.8	92.8	98.1
32	18.3	30.9	42.6	13.6	17.8	30.2	2.5	2.64	61.5	43.2	94.9	88.4
33	18.8	25.6	40.1	13.1	10.4	29.6	2.75	1.74	64.4	—	92.8	—
34	18.7	28.2	43.7	14.8	17.6	28.2	1.8	2.9	65.3	55.1	95.3	101.7
35	19.4	26.3	46.2	15.3	18.5	—	2.1	2.0	67.5	57.0	95.4	99.2
Mean	18.0	29.6	43.4	13.6	17.5	31.2	2.7	2.6	60.2	50.4	94.0	96.6
s.d.	1.76	6.82	1.77	1.02	2.14	2.97	0.57	0.62	6.3	3.5	2.1	3.8

Table 2 *Comparison of the analytical data obtained for the large and small granule fractions of rabbit liver with those reported by other investigators*

	Large granules					Small granules					Animal species
	Total lipid	Phospholipun (% dry wt)	Total N	Total P	N A P * (μg)/total N (mg)	Total lipid	Phospholipun (% dry wt)	Total N	Total P	N A P * (μg)/total N (mg)	
Claude (1946 <i>b</i>)	25	16	10-12	0.9-1.3	—	40-45	29	9.15 8.95	1.51 1.74	— —	Pig Rat
Hogeboom <i>et al</i> (1948)	—	—	—	—	15.5	—	—	—	—	58	Rat
Present author	29.6	17.5	10.5	1	70	43.4	31.2	9	1.5	80	Rabbit

(N A P =Nucleic acid phosphorus)

(N A P = Nucleic acid phosphorus)

The protein values also show considerable variation, particularly those for the large granule fraction. In the latter case, however, the variation might be due to the inhomogeneity previously mentioned. No correlation was found between the amounts of the various components in each fraction. The total lipid, nucleic acid and protein account for 94 % of the total solids of the large granules and for 96.6 % of the small granules. Thus, these fractions contain small amounts of other substances. Claude (1946b), for example, found approximately 0.5 % of inositol in the large granules. No attempt has been made in the present work to identify the unestimated components. In Table 2 the results of this investigation are compared with those of Claude (1946b) and Hogeboom *et al* (1948). The values for rabbits are in good agreement with those obtained for rats and guinea pigs. The unequal distribution of nucleic acid between the large and small granules in rat livers (Hogeboom *et al*) has not been found with rabbit liver.

Distribution of phospholipin, nucleic acid and protein between large and small granule fractions

The yield (dry weight/dry weight) of fractions from liver pulp was as follows: large granules, 3.5 ± 0.9 %, small granules, 4.9 ± 0.8 %, the ratio of

Table 3 *Distribution of constituents between large and small granules of rabbit liver*

	Large granules (%)	Small granules (%)
Nucleic acid	43	57
Protein	46	54
Total lipid	33	67
Phospholipin	29	71

the two being 0.73 ± 0.11 . If it is assumed that this is also the ratio of the granules in the liver cell cytoplasm, and if the small fractions discarded during the fractionation are neglected, then the

distribution of the cytoplasmic constituents between the two types of particles may be calculated as shown in Table 3. These values must be very approximate because of the great technical difficulties of the fractionation procedure. The distribution of phospholipin, among the large granules (1), small granules (2) and supernatant (3), was similarly calculated to be as follows (1) 26.4, (2) 64.6, (3) 9% respectively.

2 INVESTIGATIONS USING RADIOACTIVE PHOSPHORUS

The object of these experiments was to find out if there is a site in the liver cell where most of the phospholipin is synthesized. Neutral sodium phosphate containing ^{32}P was injected into rabbits and the various liver fractions prepared as described in Part 1. The specific activities (counts/min/mg) of phospholipin phosphorus of the fractions were determined, and expressed as percentage of ^{32}P injected/kg of body weight (counts/min/kg).

METHODS

Administration of ^{32}P In a few experiments of 4 hr duration, the ^{32}P (as Na_2HPO_4 in saline) was administered to the rabbits either as a continuous intravenous infusion, or in subcutaneous injections repeated at 20 min. intervals in order to maintain a constant level of inorganic ^{32}P in the plasma, as described by Hevesy & Hahn (1940). Under these conditions, the specific activities of all P compounds—including those of the liver phospholipin—should rise until they reach the level of the specific activity of the plasma inorganic P.

In the majority of experiments, however, inorganic phosphate labelled with ^{32}P was given in a single subcutaneous injection, and the animals killed 2–72 hr later. The total dose of ^{32}P administered was usually 200 μC /rabbit.

Analysis of blood Blood was obtained from the rabbits as described in Part 1. It was collected in a vessel containing heparin, cooled immediately, and the plasma drawn off after centrifugation at 2° . Approximately 15 ml. of plasma were extracted with 20 vol. of ethanol-ether (3:1, v/v) and the phospholipins obtained as described previously. Approximately 5 ml. of plasma were mixed with an equal volume of 10% (w/v) trichloroacetic acid, the precipitated protein centrifuged off and the inorganic phosphate precipitated as MgNH_4PO_4 .

Analysis of liver A sample of liver was fractionated by differential centrifugation as described in the previous section. Liver cell nuclei were prepared by the method of Mirsky & Pollister (1946) and dried from the frozen state. The phospholipins of the fractions were extracted and purified as already described, and were digested with 5% H_2SO_4 and H_2O_2 ('100 vol'). The inorganic phosphate thus obtained was precipitated as MgNH_4PO_4 .

Immediately after removal of the liver, a small portion was blotted quickly with filter paper and dropped into a freezing mixture of acetone and solid CO_2 . The frozen tissue was powdered in a steel mortar previously cooled with the freezing mixture. The crushed liver (about 5 g) was

shaken by hand with cold 5% (w/v) trichloroacetic acid (10 ml) and the mixture filtered. Inorganic phosphate was separated from the extract as MgNH_4PO_4 .

Assay of ^{32}P All P samples were obtained as dry MgNH_4PO_4 . In early experiments samples containing 50–300 μg of P were dissolved in 0.5 ml. 0.1N HCl. Of this, 0.3 ml. was pipetted accurately on to a small nickel disk, whilst 0.1 ml. was used for the determination of P. The deposits on the disks were dried by gentle heating, and the radioactivity was measured with a bell shaped Geiger-Müller counter.

Owing to the low radioactivity of some of the samples, and the difficulty of obtaining a uniform layer containing more than 300 μg of P on the disk, a cylindrical, jacketed counter, described by Veall (1948) and suitable for the measurement of radioactivity of solutions, was later used. Amounts up to 5 mg of MgNH_4PO_4 were dissolved in 1 ml. N HCl and 10 ml. of water added, the radioactivity of 10 ml. of the mixed solution was then measured and samples of the solution were used for P estimations (in duplicate).

At least 5000 and usually 10,000 counts from each specimen were taken, giving a statistical accuracy of at least $\pm 1.5\%$. The specific activity determinations are accurate to $\pm 3\%$.

RESULTS

Table 4 contains the results of an experiment in which ^{32}P was injected subcutaneously every 20 min. over a period of 4 hr. Samples of blood (5 ml) were taken at intervals after the first injection as indicated in Table 4, and the plasma inorganic phosphorus isolated as previously described. The final blood sample was taken and the liver removed 20 min. after the last injection.

Table 4 *Specific activity of plasma inorganic P and of liver P fractions after subcutaneous injection of ^{32}P*

(^{32}P injected every 20 min. over a period of 4 hr.)

A Change in plasma inorganic P with time

Time after first injection (min.)	Plasma inorganic P specific activity (counts/min/mg P)
55	77
95	77.5
135	77
175	97
215	77
235	70

B Specific activity of liver and plasma P fractions 235 min. after first injection

	Specific activity (counts/min/mg P)
Liver	
Inorganic P	39
Phospholipin P, small granules	130
Phospholipin P, large granules	114
Phospholipin P, nucleus	100
Phospholipin P, supernatant	0.73
Plasma	
Inorganic P	70
Phospholipin P	0.83

The specific activities of the various phosphorus fractions shown are typical. The specific activity of the inorganic phosphorus in the plasma is maintained

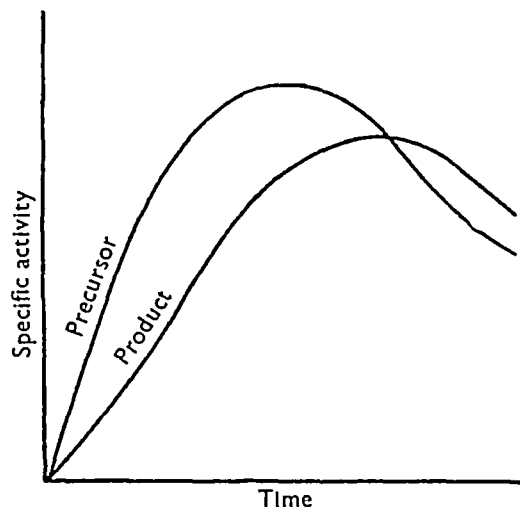


Fig 1 Theoretical relationship between the specific radioactivity of precursor and its product

at a reasonably constant level. Over the 4 hr period, the specific activity of the liver inorganic phosphorus (not corrected for extracellular inorganic phosphate) rises to about half that of the plasma inorganic value, but the uptake of ^{32}P into the phospholipin fractions

The result indicates that phospholipin fractions of different specific activities may exist in the liver cell under the conditions of this experiment. However, this experiment does not indicate whether the phospholipins in the fractions are synthesized independently of one another in the cell, or whether they are synthesized in one fraction—viz the small granules, as this fraction has the highest specific activity—and are transferred from this fraction to the others.

Zilversmit, Entenman & Fishler (1943) have established mathematically the relationship between the specific activity of a labelled precursor and that of its product in experiments in which the specific activity of the precursor is a function of time. In such experiments it is necessary to give only one injection of the labelling agent (e.g. ^{32}P). This relationship is illustrated graphically in Fig 1, from which it can be seen that the specific activity of the product at its maximum is equal to the specific activity of its precursor at the same time. Before this maximum has been reached the specific activity of the compound must be less, and afterwards it must be greater, than the specific activity of the precursor. These criteria have been employed in our experiments in which sixteen rabbits were killed at intervals of 2–72 hr after an injection of radioactive phosphate.

Since it is necessary to kill the rabbit in order to get sufficient liver for analysis, each animal can

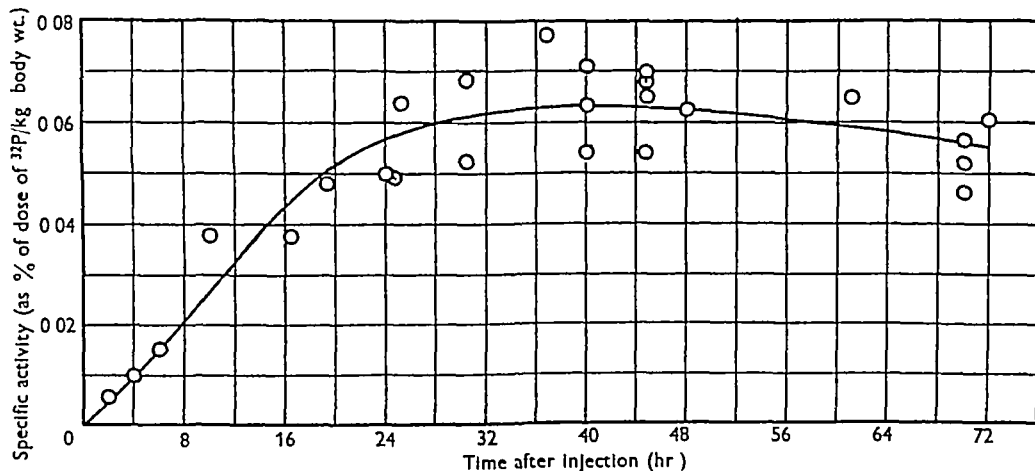


Fig 2 Change in specific radioactivity of plasma phospholipins of rabbits injected with a single dose of inorganic ^{32}P

is much slower. The two main cytoplasmic fractions, small and large granules, are similar to each other in value, with the former always slightly higher. The specific activities of the phospholipins of the nucleus and of the supernatant are somewhat lower, the value of the latter being lower than that of the plasma phospholipin.

provide data for one point only on the time curve of liver phosphorus turnover. The metabolic idiosyncrasies of rabbits are such that it is impossible to predict beforehand how much labelled phosphate to inject in order to create exactly the same level of ^{32}P in any plasma or tissue fraction in all animals at the same time. To circumvent this difficulty a

specific activity time curve of plasma phospholipins was established for an independent series of rabbits which were bled at four different intervals from 2 to 72 hr after an injection of labelled phosphate

This curve, shown in Fig 2 and reproduced again in Fig 3, was taken as the reference standard and all specific activities of the plasma phospholipin phosphorus from the main series of experiments were adjusted to fall on this curve Correspondingly, the

The first set of experiments showed that phospholipin fractions of three different specific activities existed in the liver cytoplasm, and the question arose whether the phospholipins in the fractions were synthesized separately, or whether the phospholipins in one fraction, viz the small granules, might be the precursor of the phospholipins of the others In applying the criteria laid down by Zilversmit *et al* (1943) for the establishment of a

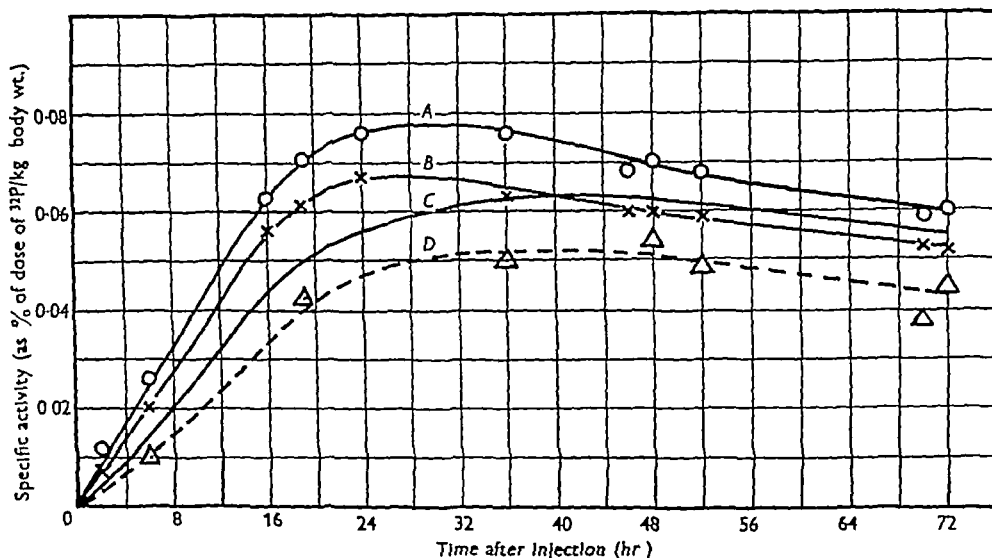


Fig 3 Change in specific radioactivity of liver and plasma phospholipins of rabbit injected with a single dose of inorganic ^{32}P A, Phospholipins extracted from small granules, B, phospholipins extracted from large granules, C, plasma phospholipins, D, phospholipins extracted from liver 'supernatant' after large and small granules have been removed.

specific activities of the three liver phospholipin fractions were multiplied by the same factor Thus this correction does not alter the ratios of the specific activities of the three liver phospholipin fractions to that of the plasma phospholipins, only the absolute values are affected

From Fig 3 it can be seen that the specific activity time curves of the three liver fractions remain independent of each other during the experiment That is, not one of these components behaves as a precursor with respect to any of the others, according to the conditions set down by Zilversmit *et al* (1943)

DISCUSSION

Hovey's (1945) original observation that the rate of renewal of phospholipin phosphorus in the liver nuclei is less than the rate in cytoplasm has been confirmed in the present series of experiments, though there does exist in the cytoplasm a small component (the supernatant) with an even slower rate of renewal

precursor, several assumptions, as recognized by these authors, are made These are (1) a steady state, in which the amount of compound present in the tissue studied must be constant during the interval over which the observations are made, (2) the rate of synthesis and degradation of the compound must be constant during the experiment, (3) the appearance and disappearance of all molecules must proceed at random, i.e the specific activity of portions of the compound breaking down (or leaving the tissues) is equal to the specific activity of the total amount of the compound present in that tissue In addition to these, a fourth assumption is involved in the present investigation of the components obtained by fractionation Since they are not simple compounds, it must be shown that their properties are reproducible from experiment to experiment The results in Table 1 show that, as far as chemical composition is concerned, this assumption may be justified

As fully matured rabbits of 2.3–2.8 kg body weight were chosen, the first and second assumptions

are justified. In the present investigation, the third assumption is perhaps not justified, as there is no evidence that the phosphorus of the phospholipin molecules distributed throughout a small or large granule is of uniform specific activity at any moment. If the latter assumption is made however, the curves in Fig. 3 indicate that phospholipin synthesis (or at least the step involving the incorporation of phosphorus into the phospholipin molecule) may take place in at least three sites in the liver cytoplasm.

It will be noticed from the curves in Fig. 3, however, that the large granule phospholipins appear to be the precursor of the plasma phospholipins. The criteria of Zilversmit *et al.* (1943) can only be applied in this case if it is known that the plasma phospholipins are entirely derived from the liver, i.e. that no phospholipin enters the plasma from any other source to alter significantly the specific activity of the total plasma phospholipins. As previously mentioned, the work of Fishler *et al.* (1943) and Entenman *et al.* (1946) has demonstrated that liver is the principal tissue in the body concerned with the production and removal of plasma phospholipins. More recently, Artom & Swanson (1948) have shown that phospholipins may reach the plasma from the intestine, though it appears that they do so only in very small amounts. If the assumption is made that the liver is the sole source of plasma phospholipins, then the curves in Fig. 3 indicate that plasma phospholipins are derived only from the large granule fraction. On the basis of this assumption, it can be calculated from the distribution figures previously presented that, under the conditions of these experiments, only 25–30% of the total phospholipin formed in the liver is transferred to the plasma. Most of the phospholipins formed in the liver remain inside the cell, and presumably are used in the normal metabolism of the cell.

It has been suggested by Claude (1943) that the large granule fraction and the microsome or small granule fraction have a common origin, or that one type of granule may contribute to the constitution of the other. Claude quotes observations which appear to support the latter possibility. While the results of the present investigation do not directly

confirm or deny these possibilities, it is significant that, as far as their phospholipin components are concerned, the metabolism of the two fractions is different, and it is therefore unlikely that one fraction is derived from the other.

SUMMARY

1. A modification of the method of Hogeboom *et al.* (1948) for the fractionation of liver cytoplasm by differential centrifugation has been used for the preparation of three fractions, large granules, small granules (microsomes) and supernatant, from rabbit liver cytoplasm. The method of Mirsky & Pollister (1946) has been used to prepare rabbit liver nuclei.

2. The microsome and large granule fractions from twelve rabbit livers have been analysed for total lipid, phospholipin, protein and nucleic acid content. Total lipid, protein and nucleic acid account for 94% of the large granules and 96.6% of the small granules. The total lipid (including phospholipin) content constitutes the main chemical difference between the two fractions, the large granules containing 29.6% and the microsomes 43.4%.

3. Approximately 26.4% of the liver cytoplasmic phospholipins was in the large granules, 64.6% in the small granules, and 9% in the supernatant.

4. The metabolism of the phospholipin phosphorus of the cytoplasmic fractions and the nucleus has been studied with the aid of radioactive phosphorus (^{32}P). It is shown that at any time after the injection of radioactive phosphate into rabbits there exist in the liver cytoplasm at least three phospholipin fractions with different specific activities. The original observation of Hevesy (1945), that the rate of renewal of phospholipin phosphorus in the liver nuclei is slower than in the cytoplasm, has been confirmed.

5. The results suggest that phospholipins are being synthesized separately and metabolized independently of each other in different morphological structures of the liver cell.

I wish to thank Dr A. S. McFarlane for his stimulating interest in this work and for valuable discussions during its progress. I am indebted to Dr G. Popják for carrying out all the operative procedures.

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Isolation of L-Threonine from Proteins

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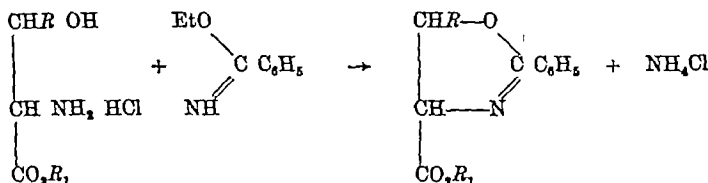
(Received 13 April 1949)

L-Threonine was first isolated from fibrin by McCoy, Meyer & Rose (1935), after a series of careful investigations extending over a number of years. It was shown to be an essential growth factor for rats. In spite of the obvious biological importance of threonine, its metabolism in animals has not been studied by the modern isotope techniques which have yielded such valuable results in the amino acid field. This is probably due to the difficulties involved in its isolation in a pure state. McCoy *et al* (1935)

properties of the hydroxyamino acids which have been briefly described previously (Elliott, 1948) and are now discussed in detail.

Separation of serine and threonine from protein hydrolysates by oxazoline derivatives

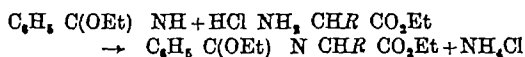
It has been shown by Elliott (1948, 1949) that serine and threonine can be converted into cyclic oxazoline derivatives by reaction of the ester hydrochloride with benziminoethyl ether in the cold. Under



worked on a very large scale and obtained only a minute yield of pure substance. Subsequently, Woolley & Peterson (1937) isolated threonine from an autolysate of the mycelium of *Aspergillus sydowii* using a different procedure. The yield of threonine was considerably higher, but in this case also the work was carried out on a large quantity of starting material. Sharp (1939) isolated a sample of threonine from rabbit myosin by fractional distillation of the esters of the 'monoamino acid fraction', but oxidation with periodate revealed that the preparation was not pure (Martin & Synge, 1941). The latter authors were able to isolate a hydroxyamino acid fraction from wool hydrolysate by their acetylation-benzoylation procedure. Pure serine was isolated from this fraction in fairly good yield, but the isolation of threonine was not attempted. Freudenberg, Walch & Molter (1942) isolated threonine from human blood group A substance. This publication has been seen only in abstract form without details.

For adequate isotope analyses and determinations of purity about 25 mg of pure threonine would be required. Owing to the small amount of threonine generally present, at least 0.5 g of protein would be required to obtain the above quantity. Since the substance sought was a minor constituent of a complex mixture considerable loss seemed inevitable, so that for these experiments were conducted with 5 g of protein. Some large scale experiments were also carried out. The method of isolation which has been worked out makes use of certain novel pro-

these conditions amino acid esters lacking the β hydroxyl group also react to give *N* substituted imino ethers (Cornforth & Cornforth, 1947)

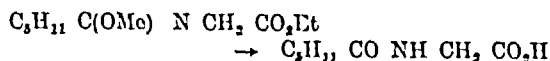


When this reaction was applied to the mixture of ester hydrochlorides resulting from the esterification of a protein hydrolysate a complex mixture resulted, this contained the oxazolines derived from serine and threonine and the substituted imino ethers from the remaining amino acids.

In this work both ethyl and isopropyl alcohols have been used for esterifying the protein hydrolysates. Synthetic studies (Elliott, 1949) have shown that the isopropyl esters give somewhat higher yields in the reaction with benziminoethyl ether.

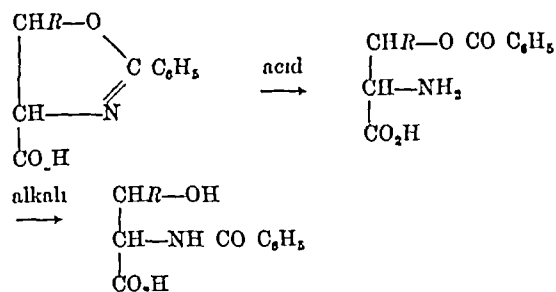
By means of a rough fractional distillation at low pressure considerable enrichment of the oxazoline fraction was effected without loss, the main contaminants being the imino ethers derived from glycine, alanine and valine. In contrast to amino acid esters, both classes of derivatives obtained by the reaction with benziminoethyl ether are quite stable to distillation and can be kept in closed vessels for several weeks without appreciable decomposition. At an early stage in the investigation it was realized that the separation of the oxazolines in a state of purity by fractional distillation would be impossible except in a fractionating column with a high theoretical plate number. Alternative methods of

purification were sought. It was shown by Abraham, Baker, Cham, Cornforth, Cornforth & Robinson (1945) that a substituted imino ether of the type formulated above was hydrolysed by alkali to the corresponding acylamino acid



Some preliminary experiments with the compound $\text{C}_6\text{H}_5 \text{ C(OEt) N CH}_2 \text{ CO}_2\text{Et}$, obtained from glycine ethyl ester hydrochloride and benziminoethyl ether, showed that the above hydrolysis was almost quantitative under suitable conditions. Although oxazolines can also be considered as substituted imino ethers it was expected that they would be more stable to alkali by virtue of the cyclic system. Studies with synthetic compounds showed that this was the case, when the oxazolines derived from serine or threonine were boiled with dilute aqueous alkali the ring system remained intact and only the carbethoxyl group was hydrolysed. This alkali treatment, when applied to the oxazoline fraction from a protein hydrolysate, was expected to produce in solution the sodium salts of the oxazoline carboxylic acids, together with the sodium salts of hippuric acid, benzoylalanine, etc. This was the case only when hydrolysis was carried out as rapidly as possible using relatively strong aqueous ethanolic alkali, milder conditions of hydrolysis led to almost complete destruction of the threonine. The reason for this is not clear, but it is probable that under alkaline conditions the esters condense with one another, the process being immediately arrested once the carbethoxyl groups have been hydrolysed.

After hydrolysis of the oxazoline fraction, the solution was acidified with hydrochloric acid to pH 1. This caused an immediate precipitation of an oily mixture of hippuric acid, etc., but the oxazoline carboxylic acids remained in solution as the hydrochlorides. Synthetic studies had established that the oxazoline ring was rapidly opened at pH 1 to give the corresponding *O* benzoyl derivative (Elliott, 1949)

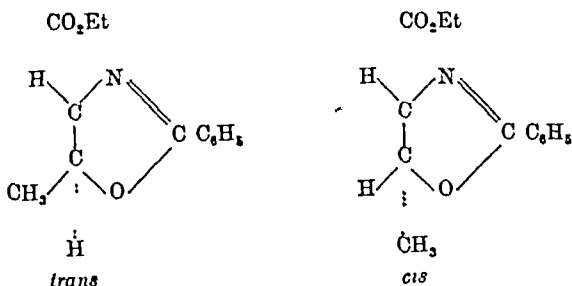


The acidified solution described above was then thoroughly extracted with ethyl acetate and the organic extract rejected. The aqueous layer contained only the *O* benzoyl derivatives of serine and threonine as their hydrochlorides, when this solution

was made alkaline to phenolphthalein the benzoyl group moved instantaneously on to the nitrogen atom. A mixture of benzoylserine and benzoylthreonine free from impurities was then obtained by a second acidification and solvent extraction. The above series of transformations can be carried out quite easily in a few hours. The remaining problem was the separation of serine from threonine, but before discussing this it is necessary to consider the stereochemical aspects of this problem.

Separation of L benzoylthreonine from DL benzoylserine

As a result of experiments, which will be described later, convincing proof has been found that benziminoethyl ether reacts with serine and threonine ester hydrochlorides to produce oxazolines with complete retention of configuration at the α (and in the case of threonine also at the β) carbon atom. It has been shown (Elliott, 1948, 1949) that oxazolines of the type under discussion most probably possess an active α hydrogen which is mobilized by the action of hot alkali. The discovery that the serine, isolated from the serine threonine mixture as described below, was completely racemized was therefore in accordance with expectations. The configuration of the threonine derivative, however, appeared to be completely preserved. The reason for this becomes obvious when the following facts are considered. Racemization at the α carbon atom in the threonine derivative would produce not the DL oxazoline, but a mixture of oxazolines derived from L threonine and D-allothreonine. These can be assigned *trans* and *cis* structures respectively, on the basis of the configurational relationship between L threonine and D threose established by Meyer & Rose (1936). These two forms must be in equilibrium in highly alkaline



solution, because of the mobile α hydrogen atom, but it has been shown (Elliott, 1948, 1949) that the equilibrium favours the *trans* form to such an extent that the *cis* form cannot be isolated.

The separation of DL benzoylserine from L benzoylthreonine was carried out in two ways. In the first method the mixture of benzoyl compounds was hydrolysed and the amino acids separated from one another by partition chromatography on a column of potato starch. The use of potato starch in partition

chromatography was first described by Syngé (1944). Although development of the chromatogram required several days, very satisfactory results were obtained on a small scale. Larger quantities of material were more easily handled by countercurrent distribution after a preliminary step in which most of the benzoylserine was removed as the sparingly soluble β phenylethylamine salt. In contrast to DL-benzoylthreonine, which also forms a sparingly soluble β phenylethylamine salt (Carter & Risser, 1941), L-benzoylthreonine forms a very soluble salt which is not crystalline. When a high yield of pure material is desirable in the separation of two substances it is advantageous to use the principle derived by Bush & Densen (1948) in which $V_z/V_y = \sqrt{1/K_a K_b}$, where V_z and V_y are the volumes of solvent used in each equilibration, and K_a, K_b are the partition coefficients of the two substances to be separated. It is also advantageous to carry the procedure through a 'diagonal stage' (see Bush & Densen, 1948, p. 123). The distribution curves of the two substances are then mirror images of each other and, providing K_a/K_b is sufficiently large, the major part of one solute will accumulate in one solvent, while the second solute will accumulate in the other solvent. The upper and lower layers can then be separately pooled and the pure components isolated from them.

In initial experiments ether and water were found to give the largest ratio K_a/K_b . The ratio of solvents to be used in each equilibration was then calculated by applying the above formula as follows. K_a (benzoylthreonine) = 0.218, K_b (benzoylserine) = 0.100, V_z = volume of ether, V_y = volume of water, $V_z/V_y = \sqrt{1/0.218} = 1/0.148$. Let p_a = the fraction of benzoylthreonine which remains in the upper phase at each equilibration. Then

$$p_a/1 - p_a = K_a V_z/V_y, \\ p_a = K_a V_z/(K_a V_z + V_y) = 0.218/(0.218 + 0.148) = 0.60$$

In the paper by Bush & Densen (1948), already referred to, a series of curves are given from which the fraction of solute in the pooled upper or lower layers can be found for all values of p and values of n (number of equilibrations) up to 100. Reference to these curves showed that a twelve funnel process should yield 80% of the original amount of benzoylthreonine from the pooled ether layers. If the bulk of the benzoylserine were removed previously as the β phenylethylamine salt this benzoylthreonine should be more than 80% pure. It was found that recrystallization from water removed the remaining impurities, but losses were considerable.

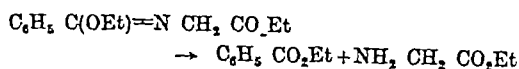
The advantage in using buffer solutions, particularly concentrated ones, in countercurrent distribution methods has been pointed out by Craig, Golumbic, Mighton & Titus (1945). After a series of experiments it was found that ethyl acetate and

a 2M-phosphate citrate buffer at pH 3.6 gave the considerably increased value of 2.9 for K_a/K_b . In this case, by calculation as above, a ten funnel process should raise the purity of the benzoylthreonine to 96%, and by repetition of this process on the purified material the purity of the benzoylthreonine should be raised almost to 100% with a loss of only 20%. Citric acid is not completely insoluble in ethyl acetate, and it was found preferable to remove this by a third countercurrent distribution of the benzoylthreonine between ethyl acetate and water. In this case K_a/K_b ($K_b = K$ for citric acid) was very high (39.5), so that the removal of citric acid was a simple matter. In practice it was found that the degree of purification was somewhat less than that calculated and the final product had a purity of 96.3%. This figure was obtained by periodate analysis of the L-threonine resulting from acid hydrolysis of the benzoyl derivative. The amino-acid had the correct specific rotation, but this did not exclude the possibility that a very small amount of D-allothreonine was present.

Fortunately, for the purpose for which this method was worked out, the optical purity of the threonine was of no consequence, it was necessary only to ensure that the product was free from other amino acids, particularly serine. Although the available evidence suggests that the threonine isolated was optically pure, this must await confirmation by a more sensitive method.

Isolation of serine from the oxazoline fraction without racemization

In order to prove that no racemization occurred when benziminoethyl ether reacted with serine and threonine ester hydrochlorides it was necessary to isolate L-serine from the oxazoline fraction by some method which avoided treatment with alkali at any stage. It has already been stated that the oxazoline ring in the compounds under discussion is readily opened in aqueous solution at pH 1. At pH 4, however, no ring opening could be detected, at least for short periods of time. Model experiments with the imino ether derived from glycine, on the other hand, showed that it was rapidly hydrolysed at pH 4 into ethyl benzoate and glycine ester. This is exactly analogous to the fission of the oxazoline ring to give the corresponding O-benzoyl compound



Oxazolines, being weak bases, should be extracted by ether from a buffer at pH 4, whereas the strongly basic amino acid esters should remain in the aqueous phase.

To test out these ideas experiments were conducted on silk sericin, which contains a very high

proportion of hydroxy amino acids (Nicolet & Seidol, 1941) This protein was obtained in solution, in partly degraded form, by a very short treatment of raw silk with hot dilute acid, the fibroin being practically unaffected The oxazoline fraction from silk sericin was shaken with an acetate buffer at pH 4 for 3 hr, the insoluble oil extracted into ether, the combined extracts evaporated and the residue hydrolysed Analyses showed that the hydroxy amino acid nitrogen content of the solution, determined by periodate ammonia estimation, was only slightly less than the amino nitrogen indicating almost complete purification of the hydroxy amino acid fraction L Serine was isolated from the hydrolysate as the *p* hydroxyazobenzene *p'* sulphonic acid salt by the method of Stein, Moore, Stamm, Chou & Bergmann (1942) and had the correct specific rotation Unfortunately, this alternative method, which seemed promising for the isolation of threonine as well as serine, gave unsatisfactory results with a casein hydrolysate and cannot be of general application

EXPERIMENTAL

Methods of analysis Serine and threonine were determined by periodate oxidation according to the method of Rees (1946) Periodate ΔH_2 was determined by the method of Van Slyke, Miller & MacFadyen (1941) The same apparatus was used for both these determinations Amino N determinations were carried out in the Van Slyke gasometric apparatus Partition coefficients were measured by equilibrating the substance between equal volumes of the two solvents and determining the amount in each layer by titration, or by Kjeldahl determinations when buffer solutions were used

Hydrolysis of the proteins A good brand of commercial casein was used for most experiments A preparation of mixed rabbit-muscle proteins was also used These were hydrolysed by refluxing 16–24 hr with 5 parts (w/v) of 6*N* HCl and were filtered from acid insoluble humin A preparation of raw silk, known commercially as 'Turkish Knubbs', was stirred with 20 parts (w/v) of boiling *N* HCl for a minute or so until the sericin had been removed This was easily judged by microscopic examination of the fibres The fibroin was then squeezed out, steeped in distilled water and squeezed out again The two solutions were combined Hydrolysis of the protein was accomplished by evaporating the solution at ordinary pressure until the acid in the distillate was about 5*N* The solution was then refluxed for 4 hr Amino N determinations revealed that hydrolysis was complete in this time In this brand of raw silk, the sericin content, as determined roughly by the weight loss on acid treatment, was 15–20%

Preliminary treatment of the protein hydrolysates Silk sericin hydrolysate required no preliminary treatment Hydrolysates from about 5 g of casein were diluted with about 5 parts of water, heated to 60° and a small amount of a concentrated solution of phosphotungstic acid stirred in This removed the acid soluble humin as a precipitate which settled rapidly Sufficient phosphotungstic acid was added to render the supernatant liquid almost colourless, but with a comparatively pure protein such as casein it was not

necessary to precipitate more than a small amount of the basic amino acids When crude proteins were used it was considered advisable to precipitate the basic amino acids almost completely, as described below After cooling, the solution was filtered through a Ford Sterimat F C B grade, extracted twice with a mixture of amyl alcohol 20 parts, ether 25 parts ethanol 1 part (see Chubnall, Rees & Williams, 1943) and evaporated to dryness under reduced pressure

On a larger scale it was preferable to remove as much as possible of the neutral, acidic and basic amino acids The following is an example of the method used The hydrolysate from 300 g of casein was evaporated under reduced pressure to about 540 ml The solution was saturated with HCl at 0° and allowed to stand 4 days at 0° To this solution was then added, with stirring, 900 ml ethanol which had previously been cooled to about -30°, the purpose of this cooling was to prevent a rise in temperature above 0° when the ethanol and the acid solution were mixed The glutamic acid hydrochloride was filtered through a sintered glass funnel and washed with 300 ml of ice cold ethanol A yield of 67 g of the hydrochloride was obtained, representing about 80% of the amount present in the protein It should be emphasized here that quantitative removal of the various fractions was not necessary The filtrate and washings from the glutamic acid were evaporated to dryness under reduced pressure, redissolved in water and evaporated again The residue was dissolved in 5.25 l of *N* HCl the solution heated to boiling and phosphotungstic acid (920 g dissolved in 1.8 l water) added fairly rapidly with stirring After standing at room temperature for 2 days the crystalline precipitate was filtered at the pump stirred with 750 ml of 2.5% (w/v) phosphotungstic acid in *N* HCl, filtered again, washed with 375 ml of the same solution on the funnel and finally sucked as dry as possible on the pump The combined filtrate and washings were saturated with ether, extracted three times with the amyl alcohol ether ethanol mixture using 2250 ml in all and then evaporated to dryness under reduced pressure This method of precipitating the basic amino acids is a simplification of the method of Chubnall *et al* (1943) The residue of amino acid hydrochlorides was then dissolved in water (750 ml), the solution cooled and 5*N* NaOH added dropwise with stirring, the temperature being kept below 15°, until the pH of the solution was about 6 Crystallization began almost immediately, and after keeping overnight at 0° the crop was filtered and washed with a small quantity of saturated NaCl solution The combined filtrates were evaporated to obtain a second crop, and so on until the crop of solid consisted almost entirely of NaCl This procedure served to remove most of the leucine and tyrosine and probably some phenylalanine and methionine The solution was then strongly acidified with conc HCl and evaporated to dryness under diminished pressure This was best carried out in a large flask because of bumping The residue was extracted thoroughly with boiling ethanol, the NaCl filtered and thoroughly washed with boiling ethanol The ethanolic extract was then evaporated to dryness under reduced pressure Analyses showed that about 20% of the threonine was lost during the above operations

Esterification of the protein hydrolysates

(a) *Ethyl esters* The syrupy residue of amino acid hydrochlorides was finally dried by evaporation twice under reduced pressure with dry ethanol The amount of ethanol used for esterification was not critical and 5–20 parts (based

on the weight of protein) were used, the amount depending on the scale. On the small scale it was more convenient to use dilute solutions. A rapid stream of dry HCl was passed through a dry ethanolic solution of the amino acid hydrochlorides until the solution boiled. It was then cooled and resaturated with HCl. After standing overnight the solution was evaporated to dryness under reduced pressure. To ensure complete esterification the process was repeated, but this may not have been necessary.

(b) *iso*Propyl esters. The residue of amino acid hydrochlorides was dried as before by evaporating with ethanol and then evaporated once with *iso*propanol. The residue was suspended in 10–20 parts of dry *iso*propanol and a rapid stream of dry HCl passed in until the solution boiled. A steady stream of dry HCl was then passed through the solution for 1 hr and boiling was maintained by application of heat at such a rate that about one third of the alcohol was allowed to escape through a 15 in. air condenser fitted to the flask. After about 30 min. fresh *iso*propanol was added to compensate for the loss through the condenser. The hydrochlorides slowly dissolved. The solution was then evaporated to dryness under reduced pressure. In the case of both (a) and (b) the esters were finally dried in a vacuum desiccator over NaOH and H_2SO_4 .

Preparation of benziminoethyl ether. Dry HCl was passed into dry ethanol (46 g) at 0° until 36.5 g. had been absorbed. The solution was then added fairly rapidly to freshly distilled benzonitrile (103 g) which had also been cooled to 0°. The mixture was allowed to stand 4 days at 0° with careful exclusion of moisture. The solid mass of imino ether hydrochloride was broken up under dry ether, filtered and washed thoroughly with dry ether. A small amount of hydrochloride separated from the ether washings on standing at 0°. The total yield was 141 g (76%), m.p. 132–134° (decomp). The hydrochloride (173 g) was added to a sludge of K_2CO_3 (129 g), and water (90 ml) which was covered with a layer of ether and cooled to –5°. After thorough shaking the ether layer was decanted and the sludge washed twice more with fresh ether. The combined extracts were dried over anhydrous Na_2SO_4 , evaporated and the residue distilled. It had b.p. 112°/22 mm. Yield 124 g (89%). The product was stable for considerable periods of time if kept in a sealed vessel.

Reaction of the ester hydrochlorides with benziminoethyl ether

The ester hydrochloride mixture from 5 g. of protein was dissolved in 3 ml. of water, and the solution adjusted to pH 3–4 by addition of a drop or two of conc. NH_4OH . A solution of benziminoethyl ether (10 g) in ether (30 ml) was then added and the mixture thoroughly shaken for 16–24 hr. Griffin and Tatlock's 'Microid' flask shaker was found to be very suitable for this purpose. The shaking was generally carried out in 250 ml. conical flasks. On a larger scale it was preferable to divide the ester hydrochloride mixture into several portions, each not greater than about 10 g. The shaker held four flasks at a time. At the end of the shaking sufficient water was added to dissolve the NH_4Cl which had crystallized out, the upper layer was separated and washed several times with fresh portions of water. It was often necessary to filter the reaction mixture after addition of water to remove amorphous material which prevented a clean separation of the layers. The ethereal layer was then dried over anhydrous Na_2SO_4 , the ether evaporated and the

residue distilled, first at about 20 mm. to remove excess of benziminoethyl ether and finally at 0.01 mm., the fraction of b.p. 90–135°/0.01 mm. being collected. This is termed the oxazoline fraction. Periodate analysis showed that it contained about 80% of the threonine originally present in the protein hydrolysate before esterification.

Hydrolysis of the oxazoline fraction isolation of a mixture of benzoylserine and benzoylthreonine

The oxazoline fraction was refluxed for 30 min. with 8 parts (w/v) of aqueous ethanolic $N NaOH$, prepared by mixing equal parts of 2N NaOH and ethanol. The solution was then cooled, diluted with about half its volume of water and the ethanol removed under reduced pressure. The solution was acidified to pH 1 with 5N HCl and allowed to stand 4 hr. at room temperature. A pale yellow oil separated. The solution was then extracted six times with about an equal volume each time of ethyl acetate, and the extracts rejected. To the aqueous solution, 5N NaOH was added until alkaline to phenolphthalein. The solution was then made weakly acid, evaporated to a small bulk and acidified (blue to congo paper) with conc. HCl. The benzoyl derivatives were obtained from the solution by six extractions with ethyl acetate. The organic extracts were combined and evaporated to dryness under reduced pressure. The yield of mixed benzoyl derivatives was about 0.35 g. from 5 g. of protein, a yield of approximately 35% (assuming a threonine content of 4% and a serine content of 6%). On a larger scale the yield was about 30%, for example, 11.84 g. of mixed benzoyl derivatives were obtained from 200 g. of casein.

Isolation of pure L-threonine and DL-serine

(a) Using a starch column

About 300 mg. of the mixture of benzoyl derivatives were refluxed 4 hr. with 3N HBr (2 ml.) and evaporated to dryness under reduced pressure. The residue was redissolved in water (20 ml.), extracted with ether to remove benzoic acid and the aqueous solution evaporated again to dryness. The residue was dissolved in 95% ethanol (2 ml.) and a slight excess of conc. NH_4OH added to the hot solution. After standing overnight the amino acid was collected and washed with ethanol. The yield from 5 g. of protein was about 80 mg. It was recrystallized from a minimum of aqueous ethanol to remove traces of NH_4Br .

Synge (1944) used unfractionated potato starch in his experiments with *n*-butanol as solvent. In the experiments about to be described phenol saturated with water was used as the solvent. It was found necessary to remove small particles from the starch by sedimentation in water until the granules, when viewed under the microscope, appeared to be of approximately equal size. This represented only about 20% of the original amount of starch. The dried product was then washed several times by stirring with aqueous phenol and separating the liquid by centrifugation. It was then allowed to soak in aqueous phenol for several days. The starch was finally made into a chromatogram 25 cm. long and 1.1 cm. in diameter. The experiment was carried out in a room with the temperature controlled to about 70° F. The column was washed by allowing aqueous phenol to percolate through it for 4 days.

About 50 mg. of the serine-threonine mixture dissolved in aqueous phenol, were added to the column, and when the solution had run through the column was developed

with aqueous phenol. The progress of the separation was followed by ninhydrin tests on a few drops of effluent. Threonine appeared in the effluent after 2-5 days and came through in a relatively small amount of solvent (12 ml., flow rate of column about 1 ml./hr.). When the ninhydrin reaction became blank or very weak the receiver was changed and washing continued to recover the serine. The amino acid was isolated from the phenol solution by adding it to an equal bulk of water and extracting the phenol twice with several volumes of ether. The aqueous solution was then evaporated to dryness and the residue crystallized from 80% ethanol. It was found that 100 mg. of amino acid mixture could be used on a column 25 x 1.8 cm. The yield of L-threonine from 5 g. of protein was 40-45 mg., representing about 20% of the amount present in the hydrolysate. Samples of amino acid isolated in this way were found to be 98-100% pure threonine by periodate analysis. No other amino acid could be detected by paper chromatography. DL-Serine isolated from the column was also pure.

(b) Using countercurrent distribution

Most of the benzoylserine was first removed from the mixture of benzoyl derivatives by dissolving 4 g. in ethanol (10 ml.) and dry ether (30 ml.), and adding freshly distilled β phenylethylamine (2.37 ml.). After standing at 0° for 2 days the crystalline DL-benzoylserine β phenylethylamine salt (2.27 g.) was collected. The filtrate from the salt was evaporated to dryness under reduced pressure, the residue was dissolved in a small quantity of water and the solution acidified to congo red. The solution was extracted six times with ethyl acetate and the combined extracts evaporated to dryness under reduced pressure to recover the crude benzoylthreonine.

Isolation of DL-benzoylserine. The β phenylethylamine salt was recrystallized several times from ethanol and then had m.p. 151-152° (Found N, 8.7. $C_{18}H_{23}O_4N_2$ requires N, 8.5%). It gave no depression in melting point on admixture with a synthetic sample of the salt. DL-Benzoylserine was obtained in good yield from the salt by dissolving it in hot water and acidifying the solution (congo red paper). The acid crystallized on cooling and had m.p. 166-167° (Found N, 6.5. $C_{10}H_{11}O_4N$ requires N, 6.7%). A 5% solution in ethanol was without effect on polarized light. About 7 g. of the β phenylethylamine salt were obtained from 200 g. of casein.

Isolation of L-threonine

Method (1) Ether/water system. About 500 mg. of the crude benzoylthreonine were submitted to a 'diagonal stage' countercurrent distribution in twelve separating funnels using 50 ml. of ether and 7.5 ml. of water in each equilibration ($K_a=0.218$, $K_b=0.100$). The pooled ether layers were then evaporated to dryness and the residue washed with light petroleum (b.p. 60-80°). The solid was recrystallized several times from a small quantity of hot water to give 150 mg. of pure L-benzoylthreonine, m.p. 143-144°, $[\alpha]_D^{25} +26.8^\circ$ in water (c, 0.7) (Found N, 6.2. Calc. for $C_{11}H_{13}O_4N$ N, 6.3%). A synthetic specimen of L-benzoylthreonine, prepared by the method of West & Carter (1937), also had the same melting point and no depression in melting point was produced on mixing the two samples. West & Carter gave $[\alpha]_D +25.1$ for their product, whereas the synthetic sample prepared in this laboratory had $[\alpha]_D^{21} +27.4^\circ$, $+26.9^\circ$ in water (c, 0.7, 0.8). An additional amount of benzoylserine was isolated from the pooled aqueous layers

as the β phenylethylamine salt. Hydrolysis of the benzoylthreonine yielded pure L-threonine (found threonine 100% by periodate analysis), which crystallized from 80% ethanol in hexagonal plates, characteristic of the pure amino acid (see McCoy *et al.* 1935; Woolley & Peterson, 1937).

Method (1) Ethyl acetate/buffer system. Phosphate citrate buffer (2M) was prepared by adding solid Na_2HPO_4 to 2M citric acid until pH 3.6 (glass electrode) was reached. The mixture was then diluted with sufficient water to make the solution 2M with respect to phosphate plus citrate. About 4 g. of crude benzoylthreonine were submitted to a 10 funnel 'diagonal stage' countercurrent distribution between ethyl acetate and the buffer, using 200 ml. of the organic solvent and 164 ml. of the buffer for each equilibration ($K_a=1.39$, $K_b=0.48$). Except for the contents of the first funnel, in which most of the coloured impurities and some benzoic acid were found, the ethyl acetate layers were pooled, evaporated and the residue submitted to a second distribution as before. Citric acid was removed from the final product by a 4 funnel process using 200 ml. of ethyl acetate and 50 ml. of water for each equilibration. The purified benzoylthreonine was crystallized once from ethyl acetate giving a product m.p. 137-141°, which was hydrolysed with HBr to give L-threonine. The yield from 200 g. of casein was about 1.3 g. (16.5% of the amount present in the casein). The amino acid was recrystallized once from aqueous ethanol to give about 0.9 g. of amino acid, $[\alpha]_D^{20} = -27.8^\circ$ in water (c, 5.0), purity by periodate analysis 96.3%. Another experiment, in which the benzoylthreonine was crystallized once from ethyl acetate before the second ethyl acetate buffer distribution, gave a sample of amino acid with $[\alpha]_D^{19} -28.2^\circ$ in water (c, 2.0), purity by periodate analysis 98.3%. West & Carter (1937) gave $[\alpha]_D^{25} -28.3^\circ$ for their synthetic sample, McCoy *et al.* (1935) found that the rotations of various isolated specimens varied from -27.5 to -28.2° .

Isolation of L-serine from silk sericin by an alternative method

A hydrolysate of silk sericin prepared from 50 g. of raw silk was esterified with isopropanol and the oxazoline fraction obtained in the usual way. The yield was 11.8 g. The main bulk (10.8 g.) of this fraction was shaken 3 hr. with 110 ml. of an acetate buffer prepared by adding solid sodium acetate to 20% acetic acid until pH 4 was reached (glass electrode). The oil which remained was extracted into ether and the extract thoroughly washed with water and $NaHCO_3$ solution. After evaporation of the ether the oil was refluxed 4 hr. with 5N H_2SO_4 (50 ml.), the solution was then extracted with ether to remove benzoic acid, diluted several times and the sulphate ions removed with $Ba(OH)_2$ in the usual way. Experiments on a small scale had revealed that the hydroxy amino acid N content of the solution as found by periodate NH_3 estimations was only 8% lower than the total amino N determined by nitrous acid. The filtrate and washings from the $BaSO_4$ were combined, evaporated to 60 ml. and *p*-hydroxyazobenzene *p'*-sulphonic acid (4 g.) added to the hot solution. On cooling the serine salt separated in the form of orange plates. It was recrystallized from hot water giving 3.2 g. of pure salt. On decomposition of the salt by the method of Stein *et al.* (1942) 0.7 g. of pure L-serine was obtained. This crystallized in heavy hexagonal prisms from aqueous ethanol. It had $[\alpha]_D^{20} -7.15^\circ$ in water (c, 3.0), and was found to be 100% pure by periodate analysis.

SUMMARY

1 Reaction of an esterified protein hydrolysate with benzaminoethylether gave a mixture of products containing the oxazolines derived from serine and threonine, and substituted imino ethers derived from amino acid esters lacking the hydroxyl group in the β position

2 Complete separation of the oxazolines from the substituted imino ethers was not attempted because of the close proximity of the various boiling points. Alkaline hydrolysis of the oxazoline fraction, followed by a series of pH changes on the resulting solution gave a mixture of DL benzoylserine and L benzoylthreonine free from other amino acid derivatives. The chemical and stereochemical changes involved are discussed

3 Serine and threonine were separated from one another on a small scale by partition chromatography on a starch column and on a larger scale, as their benzoyl derivatives, by countercurrent distribution

4 The yield of L threonine obtained on a small scale was about 20% of the amount originally present in the protein

5 L Serine was isolated from silk sericin by an alternative method which was found not to be of general application

I am indebted to Dr F O Howitt of the British Cotton Industry Research Association for generous supplies of silk and for advice, and to Dr T S Work of this Institute for his valuable help and advice on the use of starch columns. I would also like to thank Mr D Manson for expert experimental assistance

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The Distribution of Ascorbic Acid in Developing Salmon (*Salmo salar* L)

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(Received 16 March 1949)

Although attention has been focused upon physical and chemical changes that take place in the yolk and growing tissues of the developing salmon (and trout) egg, there are practically no references to vitamin content of the egg during these changes. The present investigation was limited to assays of ascorbic acid in two early embryonic stages, and one later, larval, stage of development in the salmon, and to its histological distribution. No estimations were made of the amounts of dehydroascorbic acid that might be present

METHOD

The eggs were fertilized on 1 December 1947, and kept in running water at the following temperatures: 1–15 December 1948, 9–11°, average 9.9°. This covers the period for sample 1 (9 December) and sample 2 (15 December). 1 December–9 March 1948, 7–11°, average 9.0°. This covers the period from fertilization to the date of sample 3.

Fifty eggs or larvae were taken for each sample. The method followed was that worked out by Mapson (1943). Formaldehyde was used, as first recommended by Lugg (1942), to differentiate ascorbic acid from other substances

which might interfere with subsequent titration by 2,6-dichlorophenolindophenol. Mapson (1943) studied the effects of pH, concentration of formaldehyde and time, in order to distinguish titration values for sulphur containing substances, ascorbic acid and certain non specific substances (reductones) which occur in dehydrated food. The full method was applied to the present estimations, although it is not usual to find reductones in fresh tissue.

It is difficult to estimate ascorbic acid in different parts of the fertilized egg because of the semi liquid nature of the yolk. It can be done, in a limited manner, if the yolk is first

RESULTS

With the two earlier samples the titrations showed no error due to the presence of sulphur compounds or reductones, since the values found by the method of Mapson (1943) were the same as those obtained by direct titration of a sample of the metaphosphoric acid extract with the indophenol reagent. The amount of ascorbic acid thus found was small. For

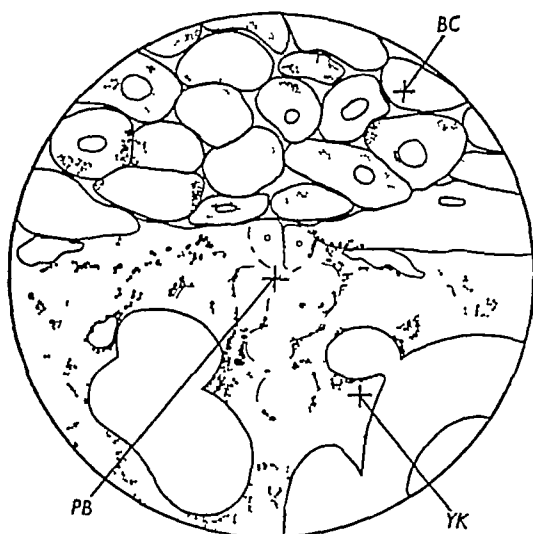


Fig 1

Fig 1 Sagittal section through blastoderm cells, periblast and yolk of salmon egg 8 days after fertilization. Objective $\frac{1}{2}$. Final magnification, $\times 288$. The black dots and masses are presumed to indicate the distribution of ascorbic acid.

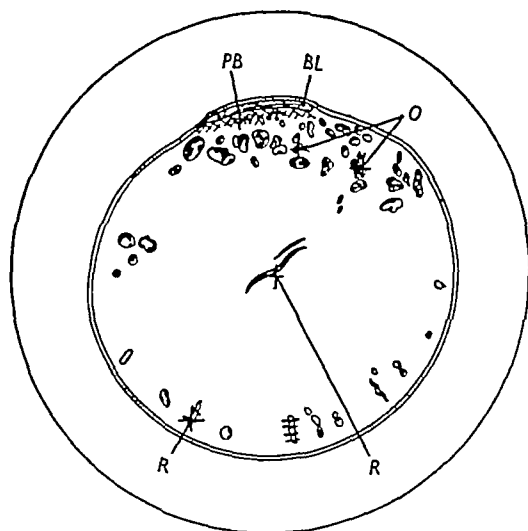


Fig 2

Fig 2 Sagittal section of salmon egg 14 days after fertilization, showing position of blackened oil globules. Objective A.A. Final magnification, $\times 94$. BC, blastoderm cell, BL, blastoderm, O, oil globules, PB, periblast, R, rifts in yolk, YK, yolk.

hardened by some fixative which also preserves the ascorbic acid. Consequently fifty eggs were taken, 19 days after fertilization, placed in a 4% (w/v) solution of HPO_3 in a closed vessel under N_2 , and kept on ice for several hours. The blastoderm, together with the underlying periblast and a small amount of yolk, was then carefully removed from each egg by means of a razor blade and treated as one sample. A second sample consisted of the rest of the yolk, yolk sac and the protoplasmic vitellin membrane which lies between the outer membrane or chorion and the yolk. In order to handle these samples as quickly as possible they were not weighed.

For the histological demonstration of ascorbic acid in the stages examined, eggs and larvae were immersed in a 10% (w/v) solution of AgNO_3 in 10% (w/v) acetic acid in total darkness (Barnett & Bourne, 1942). The AgNO_3 solution penetrates the egg envelope slowly, and the yolk is so resistant that it has to be left in the solution for several hours. The tissue was subsequently fixed in $\text{Na}_2\text{S}_2\text{O}_3$ before bringing to the light and embedding in paraffin wax for section cutting.

the sample of 9 December it was 0.0106 mg/egg of average weight 153.3 mg, i.e. 140 i.u./100 g. For 15 December, it was 0.0103 mg/egg of average weight 157.4 mg, i.e. 131 i.u./100 g.

A different result, however, was obtained from a later sample taken on 9 March, when the larvae had been hatched 50 days and the yolk almost completely absorbed. After keeping the metaphosphoric acid extract in the dark at 20° for 1 hr it was found that the titration value had fallen from 0.035 to 0.02 ml of 2,6-dichlorophenolindophenol (strength 0.1 ml equivalent to 0.04 mg of ascorbic acid). According to Mapson (1943) such a fall is due to the presence of a substance similar to dihydroxymaleic acid. Further titrations at pH 0.6 gave the same result as that obtained in the blank. That is, no ascorbic acid was present, and the reading 0.02 ml obtained in the preliminary stage was a measure of sulphur com-

pounds which had reacted to formaldehyde at the particular hydrogen ion concentration

The method used, therefore, indicated that in the two earlier samples of fertilized salmon eggs ascorbic acid was the only substance present capable of reducing 2,6-dichlorophenolindophenol. In the advanced larvae of 9 March, however, reduction was brought about by material other than ascorbic acid.

The assumption that some ascorbic acid would be retained in the tissue when eggs were fixed in a 4% solution of metaphosphoric acid, before cutting away the blastoderm, was found to be correct. The metaphosphoric acid extract and the two samples were estimated separately with the following results:

Extract	0.41 mg ascorbic acid from total wet weight of 7.432 g
Blastoderm	0.123 mg ascorbic acid
Yolk and yolk sac	0.123 mg ascorbic acid

There was an equal residue of ascorbic acid in each sample but the yolk was the bulkier sample of the two. The results show that ascorbic acid was in the yolk and the yolk sac as well as in the blastoderm and periblast, although the actual amounts are not indicated.

Examination of histological material showed that in both the early samples there had been reaction to the silver nitrate solution in the multicellular blastoderm. In prepared sections the majority of the cells contain blackened deposits of silver, either few in number or clustered together, they occur in the cytoplasm. It is not possible to connect them with yolk granules as appears to be the case in the chick (Barnett & Bourne, 1940-1), such granules are less obvious in blastoderm cells of the salmon. There was blackening, also, in the underlying protoplasmic layer of periblast. There were further deposits in the yolk immediate to the periblast where the texture is less homogeneous than in the central yolk (Fig. 1).

DISCUSSION

It is possible that substances in the tissue other than ascorbic acid reacted in a similar way to the silver nitrate solution. There is some agreement, however, between the general distribution of ascorbic acid in the egg as demonstrated by the titration method used, and the distribution of blackened granules of metallic silver in the prepared sections. It is assumed, therefore, that some of the granules, at least, result from ascorbic acid in the tissues.

Since there are no granular depositions in the yolk, other than those adjacent to the blastoderm, it may be assumed that the presence of the granules connected with activities of the blastoderm cells. This part of the yolk would be included in the estimation designated 'blastoderm' (Barnett & Bourne (1940-1)

report a like deposition in the chick, and a similar laying down of material in yolk adjacent to actively dividing cells was found in the glycogen content of developing salmon (Daniel, 1946).

Although it has been demonstrated, by titration, that ascorbic acid is also present in the rest of the yolk sac, it is difficult to form an opinion from histological preparations about its distribution. There is a general tendency for metallic silver to be precipitated wherever there are rifts or interfaces in the yolk, even in tissue that has been fixed for short periods (Fig. 2). Incidentally, there are similar deposits in sections that were passed through water and ethanol before being immersed in silver nitrate solution. They occur also in the late larval stage of 9 March, although quantitative analysis indicated that no ascorbic acid was present.

It is possible that masses of blackened material which lie in many oil vacuoles of the yolk may be connected with the presence of ascorbic acid, since the latter is not manifest in the surrounding yolk itself. Conditions under which a water-soluble vitamin, such as ascorbic acid, could be allied with oil globules call for further examination. In the meantime, two possibilities are presented.

(1) *Attraction of ascorbic acid to the oil globules*. This suggestion is prompted by an experiment of Barnett & Fisher (1944) with 5% gelatin, drops of olive oil and ascorbic acid to give a concentration of 200 mg/100 ml. The mixture was emulsified and pieces left in acidified silver nitrate for 10 min. Examination under the microscope showed that some of the oil globules had a heavy precipitate of metallic silver attached to the surface. The investigators claimed that this localization is a physico-chemical phenomenon occurring after the precipitation. Their figure, however, shows that the blackening is almost wholly confined to the surface of the oil globules, and is different in kind from that found under somewhat similar conditions in yolk oil globules (Fig. 2).

(2) *Affinity between cholesterol and ascorbic acid*. Recent work on the adrenal gland has suggested an affinity between cholesterol and ascorbic acid (Long, 1947). It has been put forward by Zwemer, Lowenstein & Pines (1940) that the adrenal gland contains water-soluble steroids which are associated with ascorbic acid. Long states that approximately 90% of adrenal cholesterol is present in ester form. According to M. Scott (unpublished), only free cholesterol is present in oil obtained from yolk of developing salmon larvae. Conditions therefore would appear to be even more favourable for a linkage with ascorbic acid.

Finally, there may have been reaction between the silver nitrate solution and the original oil content of the vacuoles, in fact the amount of material involved suggests this to be the case. Smears of highly

unsaturated cod liver oil, which is free from ascorbic acid, shows an intense black deposit after immersion in acidified silver nitrate in the dark (although not *in situ* in the liver), and to a limited extent this is also true of oil removed from salmon yolk

In the late larval stage of 9 March, the various organs are almost completely differentiated. Although no ascorbic acid was found by estimation at this stage, there appears to be some deposition of metallic salts in the tissue. In section the issue is confused by the presence of black pigment, but there are certain discrete deposits near the vertebral column and in cells of the yolk sac

SUMMARY

1 It was found by the titrimetric method of Mapson (1943) that ascorbic acid was present in early

stages of developing salmon (8 and 14 days after fertilization), but absent in a late larval stage in which most of the yolk had been absorbed. It was contained both in the blastoderm region and in the yolk and yolk-sac

2 Sections obtained from material fixed in acidified silver nitrate show that ascorbic acid may be present in blastoderm cells, in the underlying periblast and also in adjacent yolk. In this respect the distribution is much the same as in early stages of chick development as recorded by Barnett & Bourne (1940-1)

3 There are intense deposits of metallic silver where oil globules have been situated in the main part of the yolk. These may be due, in part, to ascorbic acid present in the yolk, although there is also evidence in favour of a reaction between the silver nitrate solution and the oil globules

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Studies in Biochemical Adaptation. Effect of Diet on the Intestinal Phosphatase of the Rat

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(Received 14 April 1949)

Verzár & McDougall (1936) suggested that the absorption of glucose and fructose is accompanied by phosphorylation by the intestinal mucosa. Part of the evidence upon which this theory was based was that iodoacetate decreases the rate of absorption of glucose, and this evidence has been criticized by several later writers (e.g. Klinghoffer, 1938). Again, Verzár supposed that an adrenal cortical hormone was responsible for the phosphorylation, and this has been criticized (Cori & Cori, 1941). Nevertheless, most workers accept the view that phosphorylation of glucose and fructose is an important stage in their absorption.

It has also been suggested that fats are phosphorylated before absorption (e.g. Sinclair, 1929). Verzár & McDougall (1936) claimed that this was controlled

by the adrenal cortex, but, as with their theory of carbohydrate absorption, this view has since been severely criticized. Frazer (1948, see also Frazer, 1946) has suggested that fat may be absorbed as the unchanged triglyceride, although this is not universally accepted (Lundbaek & Maaloe, 1947). Most workers are agreed, however, that at least some phosphorylation of fat occurs during absorption (Schmidt Nielsen, 1946).

It might be considered that diets with varying proportions of carbohydrate and fat might possibly stimulate the small intestine to produce varying amounts of phosphatase, since it is known that the amounts of some digestive enzymes are affected by variations in the degree to which they are called upon to carry out their work. A study was therefore

made of the amount of intestinal phosphatase in animals fed on diets containing different amounts of carbohydrate and of fat

EXPERIMENTAL

Piebald rats of the Hartwell strain were placed on one of four diets for 6-7 months from weaning. The diets were constructed so as to contain varying proportions of protein, carbohydrate and fat (Table 1). Each diet was fed to ten rats, five male and five female, each rat being housed in a separate cage.

Table 1 *Composition of diets*

Diet	Casein (‘Light white’ Glaxo) (%)	Sucrose (%)	Lard (%)
Basal	15	55	15
Fat-free	15	70	0
High fat	15	0	70
High protein	70	15	0

In addition, each diet contained dried yeast (10%) and salt mixture (5%). Each animal was given cod liver oil (5 drops twice weekly).

Preparation of intestinal extract. Animals were killed by exsanguination. The small intestine was dissected out, as free as possible from adherent tissue, from pylorus to caecum. The contents were removed by blowing a gentle stream of saline through the lumen. Since the activity of phosphatase decreases progressively from duodenum to caecum (Kay, 1932, Folley & Kay, 1936), it was thought desirable to assay the enzyme separately in the proximal and distal portions of the intestine. The intestine was therefore divided into as nearly as possible equal proximal and distal lengths. Each length was slit open, dried gently between filter paper and weighed. It was then ground with about twice its weight of washed silver sand. Water saturated with CHCl_3 was added to a volume exactly ten times the original wet weight of the intestine and the suspension kept for 24 hr. at laboratory temperature with occasional shaking.

Estimation of phosphatase activity. The alkaline phosphatase was assayed separately in the extracts from the proximal and distal parts of each intestine. The extracts, after standing for 24 hr., were further diluted with CHCl_3 water so that they contained 1 g. of wet tissue in 1000 ml. for the proximal part of the intestine and 1 g. in 250 ml. for the distal part. These dilutions were found to give enzymic activities such that conveniently measurable amounts of phosphate ions were liberated in the period of incubation with the substrate.

The phosphatase digest was set up as follows. 1 ml. of the diluted extract was added to 10 ml. of buffered sodium β glycerophosphate (0.5 w/v), and the mixture incubated at 37° for 1 hr. Of this mixture, 1 ml. was added to 4 ml. of water, to this was added 5 ml. of trichloroacetic acid (12% w/v) and the whole well mixed. It was not necessary to centrifuge or filter at this stage since, owing to the high dilution of the enzymic preparation, the amount of protein precipitated was so little that no significant turbidity occurred.

Inorganic phosphate was estimated in the preparation by placing 5 ml. in a colorimeter tube adding 4 ml. of acid molybdate reagent and 1 ml. of freshly prepared dilute

SnCl_2 solution, the colour being measured in the Evelyn photoelectric colorimeter after 10 min. at laboratory temperature. An exactly similar analysis, carried out upon a 1 ml. sample of the digest taken immediately after adding the enzyme preparation, gave the initial inorganic phosphate content of the digest. The solutions of sodium β glycerophosphate, molybdate and SnCl_2 were prepared as described by Bodansky (1932-3).

The activity of the enzyme is expressed in ‘units’ (mg. P liberated/g. tissue/hr. at pH 9.2 and 37°). No attempt was made to obtain possible activation of the enzyme by, for example, the addition of salts of Mg or Zn.

RESULTS

The mean weights of the animals and of the whole of their small intestines are given in Table 2. The differences between the different groups of animals

Table 2 *Effect of diet on weight of rat and of small intestine*

Diet	Average wt. of rats (g.)	Average wet wt. of small intestine (g.)
Basal	187	6.45
Fat-free	186	6.74
High fat	188	6.45
High protein	193	6.76
Average, all groups	184	6.62

are statistically not significant. As was to be expected, the average weights of the female animals and of their small intestines were lower than those of the males. Also, the average weights of the proximal portions of the intestines were higher than those of the distal portions. Table 3 shows these values for all groups combined.

Table 3 *Weight of rats and of small intestine. Comparison of male and female rats, and of proximal and distal portions of intestine*

	Average wt. of rats (g.)	Average wet wt. of small intestine (g.)		
		Whole	Proximal portion	Distal portion
Male	202	7.40	4.03	3.37
Female	166	5.83	3.17	2.66
Average, all rats	184	6.62	3.60	3.02

The values for phosphatase activities are given in Table 4. It seems reasonable to conclude that there is the same amount of intestinal phosphatase in the rats on the basal and fat-free diets and also the same amount in the rats on the high fat and high protein diets. On the other hand, there is more intestinal phosphatase in rats on either of the latter two diets than in those on either of the former two diets. For example, rats on the basal and fat-free diets had intestinal phosphatase values of 52 and 58 units/g., rats on the high fat and high protein diets had values of 77 and 78 units/g.

Table 4 *Phosphatase activity of small intestine of rats on different diets*(Ten rats on each diet Results (mean \pm s.e.) expressed as 'units', i.e. mg P liberated in 1 hr at pH 9.2 and 37°)

Tissue		Diet			
		Basal	Fat-free	High fat	High protein
Whole intestine	Total units	(1) 330 \pm 46.5	(2) 391 \pm 41.7	(3) 489 \pm 39.3	(4) 538 \pm 42.6
	Units/g	(5) 52 \pm 7.1	(6) 58 \pm 4.8	(7) 77 \pm 6.3	(8) 78 \pm 6.4
Proximal portion	Total units	(9) 271 \pm 40.7	(10) 328 \pm 31.3	(11) 375 \pm 33.0	(12) 448 \pm 37.2
	Units/g	(13) 77 \pm 10.6	(14) 87 \pm 6.0	(15) 113 \pm 8.3	(16) 119 \pm 10.9
Distal portion	Total units	(17) 58 \pm 7.8	(18) 62 \pm 13.3	(19) 114 \pm 10.8	(20) 90 \pm 16.1
	Units/g	(21) 20.3 \pm 3.4	(22) 20.3 \pm 3.9	(23) 34.5 \pm 3.5	(24) 29.0 \pm 3.7

Significant differences ($P < 0.05$) exist between the following groups (1) and (3), (1) and (4), (2) and (4), (5) and (7), (5) and (8), (6) and (7), (6) and (8), (9) and (11), (9) and (12), (10) and (12), (13) and (15), (13) and (16), (14) and (15), (14) and (16), (17) and (19), (17) and (20), (18) and (19), (21) and (23), (22) and (23)

DISCUSSION

The chief difference between the basal and fat free diets on the one hand and the high fat and the high protein diets on the other is the presence of much carbohydrate (55 or 70 % sucrose) in the former and its absence in the latter. It would seem therefore that the presence of sucrose leads to a reduction in the amount of phosphatase in the intestinal tissue. References to similar work are very meagre. Bellini & Cera (1940) found an increase in the intestinal phosphatase of the rat during fat absorption. Weil & Russell (1940) found a decrease in plasma phosphatase in the rat during fasting, and an increase during the feeding of lard and of cephalin but not of lecithin. Lundbaek & Goranson (1948) showed that the

phosphorylase of rat muscle is increased during starvation.

We can at present suggest no reason why the presence of sucrose in the diet should lead to a decrease in intestinal phosphatase, and until more data are available it is profitless to speculate.

SUMMARY

The effect was studied of varying the proportions of dietary protein, fat and carbohydrate on the alkaline phosphatase of the small intestine of the rat. Diets containing 55 or 70 % of sucrose produced a significantly lower amount of the enzyme than diets in which the sucrose was replaced by fat or protein.

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The Reactions of Haems with Cyanides and isocyanides

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(Received 2 May 1949)

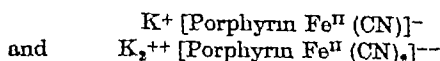
By determining the minimum amount of pyridine required to give a haemochromogen with a very strong solution of haem (ferrous protoporphyrin), Hill (1926) demonstrated conclusively that pyridine haemochromogen contains 2 molecules of organic base per molecule of haem or per atom of iron. This result was in agreement with that of von Zeynek

(1910) who found 2.2 mol. of pyridine per mol. of haem in a solid pyridine haemochromogen.

Potassium cyanide, however, unlike other nitrogenous substances, was found by Anson & Mirsky (1928) to give two distinct compounds with haem. The first, containing 1 mol. of cyanide per molecule of haem, was named by them 'cyan-haemochromogen'.

on account of the resemblance of its absorption spectrum to that of pyridine haemochromogen. The second compound, containing more cyanide per molecule of haem, was merely named the 'second cyanide compound'. The latter, which can only be obtained in presence of an excess of cyanide, differs from the first in the general pattern of its absorption spectrum. Unlike a typical haemochromogen its β band is much stronger than its α band. (Following general usage the letters α , β and γ denote the individual bands of the absorption spectrum of a compound, proceeding in that order from the red towards the ultraviolet regions of the spectrum. In haematin compounds the α - and β bands are in the visible region, while the γ band, also known as the Soret band, lies in the violet part of the spectrum.) These facts led Anson & Mirsky (1928) to consider all typical haemochromogens as compounds of haem with 1 mol. of a nitrogenous base, a conclusion which was not corroborated by later work. The nature of the compounds of cyanide with haem was reinvestigated by Hill (1929), who confirmed Anson & Mirsky's finding that haem and cyanide may combine in equimolecular proportions to give a compound with a haemochromogen like spectrum, especially if the solution has a relatively low concentration of salts which tend to precipitate the haem out of solution. He also showed that the second compound, which had previously been obtained in the presence of excess cyanide, contains only 2 mol. of cyanide per molecule of haem, and he was able to determine the dissociation constants of both compounds.

As it is the 'second cyanide compound' of Anson & Mirsky (containing 2 molecules of cyanide per molecule of haem) that is structurally analogous to pyridine haemochromogen, Hill described it under the name 'cyan haemochromogen' whereas for the monocyano compound he proposed the name 'cyan reduced haematin'. Since, as will be shown later, both these cyanide haem compounds differ in several respects from a typical haemochromogen, the protohaem compounds containing 1 and 2 mol. of cyanide will be described as monocyano-haem $[(CN^-) \text{ haem}]$ and dicyano haem $[(CN^-)_2 \text{ haem}]$ respectively. Suggested structures for these two compounds are



In this paper it is proposed (1) to give the absorption spectra in the visible and violet regions of monocyano and dicyano haem, (2) to answer the question raised by Hill (1926) as to the existence of a compound in which the haem iron is linked simultaneously to the cyanide radical and carbon monoxide, (3) to elucidate the mode of attachment of the cyanide ion to the haem iron, since it is theoretically possible for the haem iron to combine with either

the nitrogen or the carbon atom of the cyanide radical. For this the reactions of non dissociable cyanides and isocyanides with haems, haematin and methaemoglobin were examined.

EXPERIMENTAL

Protohaemin This was prepared by the method of Schälfeff (1885) from ox or horse blood.

Urohaemin Uroporphyrin I was isolated from the urine of a case of congenital porphyria. The porphyrin was esterified and the urohaemin prepared from the octamethyl ester according to Fischer & Orth (1934).

Other reagents KCN solutions in distilled water were standardized either by the method of Robbie & Lemfelder (1945) or with standard $AgNO_3$ in the usual manner. Methyl cyanide was purified by adapting the method of Toda (1926) for the purification of butyl cyanide. Methyl isocyanide was prepared according to the method of Gautier as modified by Hartley (1928).

Spectroscopic observations For all qualitative spectroscopic examinations and for preliminary quantitative experiments a microspectroscope was used as previously described (Keilin, 1943). The detailed study of absorption spectra was carried out with a Beckman photoelectric spectrophotometer using 0.5 and 0.25 cm. cells. The absorption coefficient is defined as $\epsilon = d/cl$, where c = molarity of the haematin solution, l = optical depth and d (density) = $\log I_0/I$, where I_0 and I are the intensities of the incident and transmitted light, respectively.

RESULTS

Monocyano haem

A stock solution of protohaematin ($1.8 \times 10^{-4} M$) was prepared by dissolving 11.7 mg. crystalline protohaemin in 100 ml. 1% (w/v) (anhydrous) Na_2CO_3 . Monocyano haem $[(CN^-) \text{ haem}]$ was obtained by mixing 1 ml. of the stock haematin solution with 0.5 ml. of $3.6 \times 10^{-4} M$ aqueous solution of potassium cyanide, the mixture being made up to 6 ml. with water and reduced with a few mg. of solid sodium dithionite ($Na_2S_2O_4$). In this solution the haem and cyanide were present in a 1:1 molecular ratio, the concentration of each being $0.3 \times 10^{-4} M$. The absorption spectrum of the scarlet solution of this compound shows two distinct bands in the visible region α , 552 and β , 522 $m\mu$, the α band being much stronger than the β band. The γ band is at 414 $m\mu$. The degree of dissociation which occurred under the conditions of this experiment was calculated from the value of the dissociation constant $K = 1.3 \times 10^{-6}$ at 16°, given by Hill (1929). It was found that only 52.4% of the total haem was present as $(CN^-) \text{ haem}$, 47.6% remaining as free haem. These figures are in agreement with Hill's dissociation curve of $(CN^-) \text{ haem}$ where cyanide and haem are present in a 1:1 molecular ratio. The absorption curve of $(CN^-) \text{ haem}$, as measured under the above experimental conditions, and the theoretical

curve of the absorption spectrum for 100% (CN⁻)-haem, which was calculated from the observed values, are shown in Figs 1 and 2

The extinction coefficients of the α , β and γ bands, measured and theoretical, are summarized in Tables 1 and 2

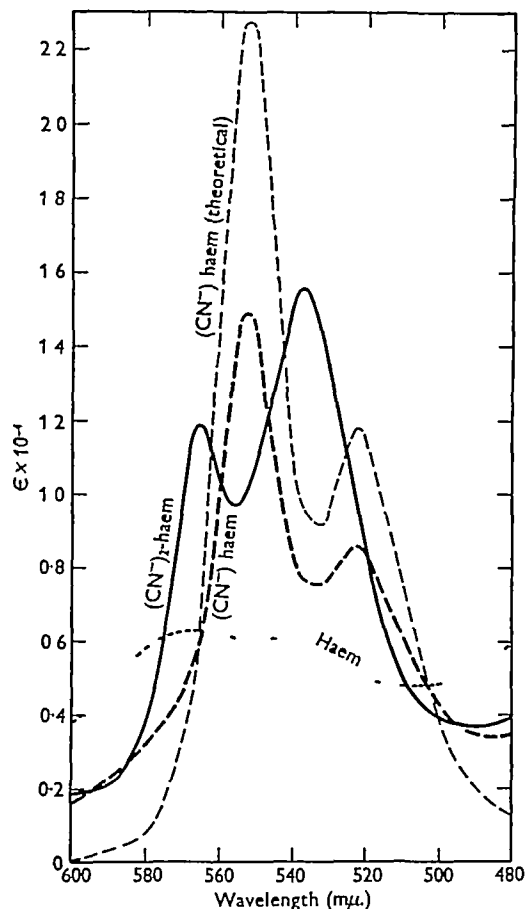


Fig 1 Absorption bands in the visible region of protohaem, (CN⁻) protohaem and (CN⁻)₂ protohaem. Haem = 0.3×10^{-4} M, $l = 0.5$ cm, ϵ as defined in text. The theoretical absorption curve of (CN⁻) haem calculated for the undissociated compound is also given

The absorption spectrum of this compound is of special interest in that although it is of a haemochromogen pattern, its α - and β bands are considerably lower than those of typical haemochromogens obtained with denatured serum proteins, denatured globin, glycine or pyridine (Table 1). However, in the case of (CN⁻) urohaem (Fig 5) it was found that the α -band was very nearly as high ($\epsilon \times 10^{-4} = 1.99$) as that of a typical urohaemochromogen ($\epsilon \times 10^{-4} = 2.23$ for glycine urohaemochromogen).

So far, the magnetic susceptibility of (CN⁻) haem has not been determined owing to technical difficulties. As has already been pointed out by Hill

(1929), the strongest solution of (CN⁻) haem that can be obtained is 2×10^{-4} M. Even though the haem and cyanide are present in 1:1 ratio, a more concentrated solution would contain free haem in equilibrium with dicyan-haem. For the accurate determination of the magnetic susceptibility of a haematin derivative by the method of Gouy (1889) using a magnetic field of about 10,000 gauss, the concentration of the haem should be at least 10^{-2} M.

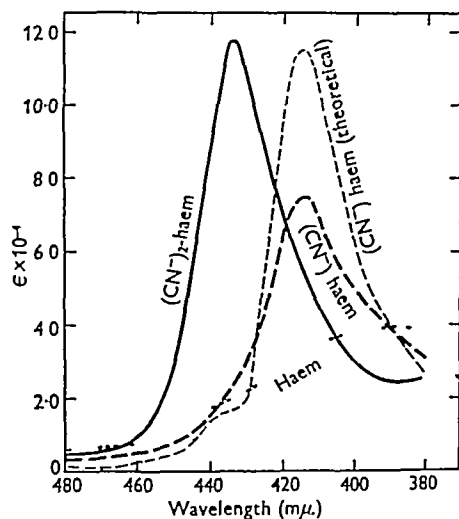


Fig 2 Absorption spectra showing γ bands of protohaem, (CN⁻) protohaem and (CN⁻)₂ protohaem, and the theoretical absorption curve of (CN⁻) protohaem calculated for the undissociated compound. Haem = 0.3×10^{-4} M, $l = 0.5$ cm

Dicyan haem

Dicyan haem, [(CN⁻)₂ haem], was prepared by mixing 1 ml of the stock haematin solution with 5 ml of 3.6×10^{-3} M aqueous potassium cyanide solution reduced with the minimum amount of dry Na₂S₂O₄. The concentration of haem was 0.3×10^{-4} M and the haem cyanide ratio was 1:100. The absorption spectrum of the solution of this compound, which is also scarlet, differs markedly from that of (CN⁻) haem (Figs 1 and 2). The positions of its two absorption bands in the visible region of the spectrum are α , 565 and β , 536 mμ, and the α band is much weaker than the β -band, the values $\epsilon \times 10^{-4}$ being 1.185 and 1.56, respectively. The γ band lies at 434 mμ. The extinction coefficients of the absorption bands are summarized in Table 2.

Reactions with carbon monoxide

Reactions of haemochromogens with carbon monoxide
It is well known that haem and haemochromogens combine reversibly with CO and that the CO haem and CO-haemochromogens thus formed each contain only 1 mol of CO/mol of the compound

Table 1 Positions and heights of the α and β bands and the trough between them for monocyano protohaem and typical haemochromogen compounds

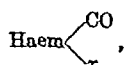
Compound	Wavelength of maxima and minima of absorption		$\epsilon \times 10^{-4}$	$\frac{\epsilon_{\alpha}}{\epsilon_{tr}}$	$\frac{\epsilon_{\beta}}{\epsilon_{tr}}$
	Band or trough (tr)	(m μ)			
Monocyano protohaem (theoretical value)	α	552	2.27	2.5	1.3
	tr	534	0.91		
	β	522	1.18		
Denatured serum protein haemochromogen (Keilin, 1944)	α	557	2.96	3.76	1.64
	tr	540	0.79		
	β	527	1.29		
Glycine haemochromogen (Keilin, unpublished)	α	556	3.02	3.65	1.71
	tr	539	0.83		
	β	525	1.30		
Globin haemochromogen (Drabkin, 1942)	α	558	3.09	3.49	1.58
	tr	542	0.89		
	β	528	1.40		
Pyridine haemochromogen (Drabkin, 1942)	α	558	3.09	3.41	1.79
	tr	540	0.91		
	β	525	1.63		

Table 2 Positions and extinction coefficients of the α , β and γ -bands of the compounds of protohaem and urohaem with KCN, CH_3NC and CO

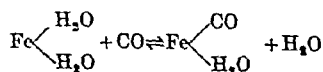
Compound	α Band		β Band		γ Band		$\frac{\epsilon_{\gamma}}{\epsilon_{\alpha}}$
	λ (m μ)	$\epsilon \times 10^{-4}$	λ (m μ)	$\epsilon \times 10^{-4}$	λ (m μ)	$\epsilon \times 10^{-4}$	
(CN $^-$) protohaem (theoretical)	552	2.27	522	1.18	414	11.45	5
(CN $^-$) protohaem (exp)	552	1.49*	522	0.86*	414	7.5*	5
(CH $_3$ NC) protohaem	556	1.515*	528	1.13*	420	10.45*	7
(CN $^-$) $_2$ protohaem	565	1.185	536	1.56	434	11.75	10.0
(CH $_3$ NC) $_2$ protohaem	562	1.245	532	1.415	432	14.48	11.6
CO protohaem	562	1.46	530	1.195	406.5	14.7	9.6
(CN $^-$) protohaem + CO	562	1.40	530	1.15	406.5	14.1	10.0
CO cyan protohaem	565	0.97	538	1.185	425	15.0	15.4
CO protohaem (in ethanol)	562	1.185	533	0.92	410	15.6	13.2
CO (CH $_3$ NC) protohaem	564	1.205	536	1.155	422.5	10.0	8.3
(CN $^-$) urohaem	547	1.99*	518	1.17*	418	11.2*	5.6
(CH $_3$ NC) urohaem	547	1.655*	516	1.185*	410	17.5*	10.5
(CN $^-$) $_2$ urohaem	560	0.83	532	1.46	428	11.0	13.25
(CH $_3$ NC) $_2$ urohaem	554	1.1	524	1.55	422	26.8	24.5

* These values apply to the compound under the experimental conditions described and are much lower than the theoretical value owing to dissociation

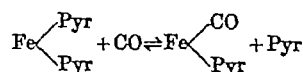
(Hill, 1926) According to Hill CO haem can be represented as



'where x is either another molecule of the complex or a molecule of water' (In the schematic representations of these compounds, the lines directed from the haem iron are not intended to indicate the nature of the valency bonds) Using the formula for haem suggested by Davies (1940) the reaction may be expressed as follows (Fe = haem iron)



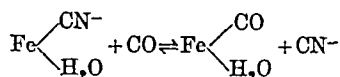
By passing CO through a solution of a haemochromogen, e.g. pyridine haemochromogen, the CO displaces 1 mol of pyridine (Pyr) to form a CO haemochromogen (Hill, 1926)



It is important to note that in this equilibrium reaction CO and pyridine compete for the haem iron

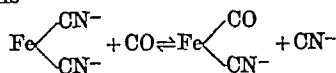
Reaction of monocyano haem with carbon monoxide On passing CO through a solution of (CN $^-$)-haem, the latter acquires a characteristic bright cherry-red colour, and its absorption spectrum in both the visible and violet regions becomes almost

indistinguishable from that of ordinary CO haem. To avoid loss of cyanide as CO is bubbled through the solution, the experiment was also carried out by adding an equivalent amount of cyanide to CO haem previously prepared. However, the same result was obtained in both cases and the compound formed could not be distinguished from CO haem. These results support the observation of Hill (1929) that when (CN^-) haem is treated with CO the latter replaces the single cyanide ion giving CO haem and not CO (CN^-) haem. The reaction may therefore be represented as follows:



Reaction of dicyan haem with carbon monoxide

When CO was passed through a solution of $(\text{CN}^-)_2$ -haem, the characteristic absorption bands of this compound in the visible region of the spectrum disappeared and were replaced by a diffuse and asymmetric band as seen in the microspectroscope. The spectrophotometric curve of this band (Fig. 3), however, showed it to consist of a two-banded spectrum with a very weak and narrow band at $565 \text{ m}\mu$ and a strong band at $538 \text{ m}\mu$. There was also a very marked change in the violet region of the spectrum, the γ band of the compound became distinctly sharper and was shifted from 434 to $425 \text{ m}\mu$, i.e. about $9 \text{ m}\mu$ towards the short wave end of the spectrum (Fig. 4, Table 2). The absorption spectrum of this compound is thus very different from those of (CN^-) -haem, $(\text{CN}^-)_2$ haem, CO haem and CO haemochromogen. These results clearly demonstrate the existence of CO cyan haem, which may be considered as analogous to CO haemochromogens.



Carbylamine haems

Reactions of methyl isocyanide with haems Pure methyl isocyanide (CH_3NC) was dissolved in distilled water to make a 0.15 M solution. A 10^{-3} M solution of protohaematin was prepared by dissolving 16.4 mg of crystalline protohaemin in 25 ml 0.1 N NaOH. The isocyanide solution (1 ml) was added to 1 ml haematin solution, and the mixture was diluted with methanol and distilled water so that the final methanol concentration was at least 50% . When this solution, containing haem and isocyanide in a $1:150$ molecular ratio, was reduced with $\text{Na}_2\text{S}_2\text{O}_4$ the red solution showed a two-banded spectrum resembling that of $(\text{CN}^-)_2$ -haem, the β band being sharper than the α band. The positions of the bands were α , 562 , β , 532 and γ , $432 \text{ m}\mu$, the values of $\epsilon \times 10^{-4}$ being 1.25 , 1.42 and 14.48 , respectively (Table 2). A similar experiment using equimolecular

(10^{-3} M) solutions of haematin and isocyanide gave rise to a spectrum resembling that of (CN^-) haem in

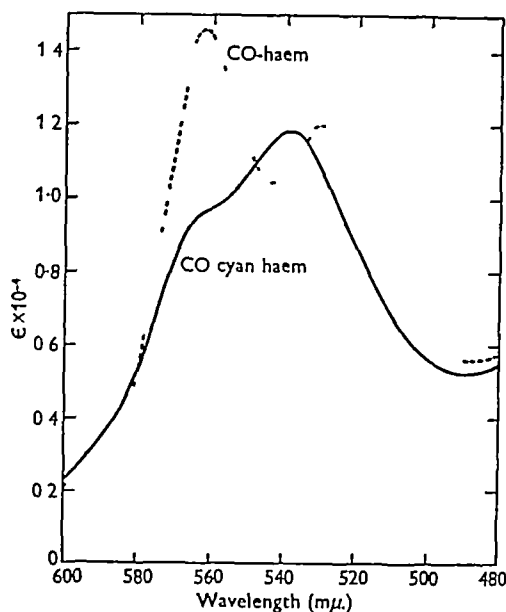


Fig. 3 Absorption bands in the visible region of the spectrum of CO protohaem and CO cyan protohaem. Haem = $0.3 \times 10^{-4} \text{ M}$, $l = 0.5 \text{ cm}$.

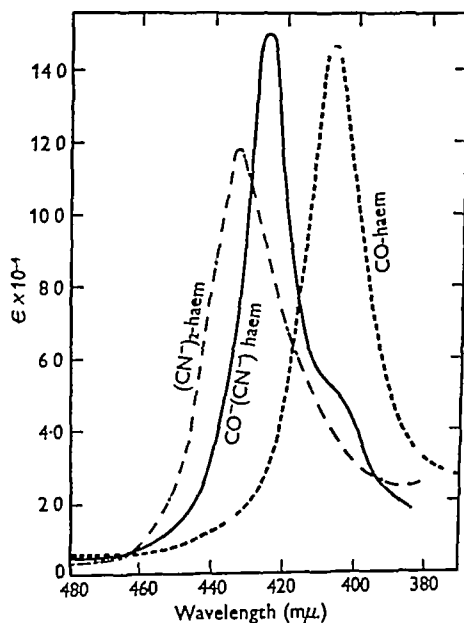


Fig. 4 Absorption spectra showing γ bands of CO protohaem, $(\text{CN}^-)_2$ protohaem and CO cyan protohaem. The shoulder at about $405 \text{ m}\mu$ on the latter absorption band is probably due to a little CO haem in the solution. Haem = $0.3 \times 10^{-4} \text{ M}$, $l = 0.5 \text{ cm}$.

which the α band was much sharper than the β band. Owing to some dissociation under the conditions of the experiment the absorption spectrum is

probably that of a mixture of (CH_3NC) haem with a little free haem and $(\text{CH}_3\text{NC})_2$ haem, the latter giving rise to a low γ band in addition to that of (CH_3NC) haem. The positions and extinction coefficients of the absorption bands of this solution are summarized in Table 2.

With urohaem, CH_3NC reacts freely in slightly alkaline solution, presumably because urohaem is so much more soluble than protohaem. Two compounds are formed as with protohaem, and the spectra are

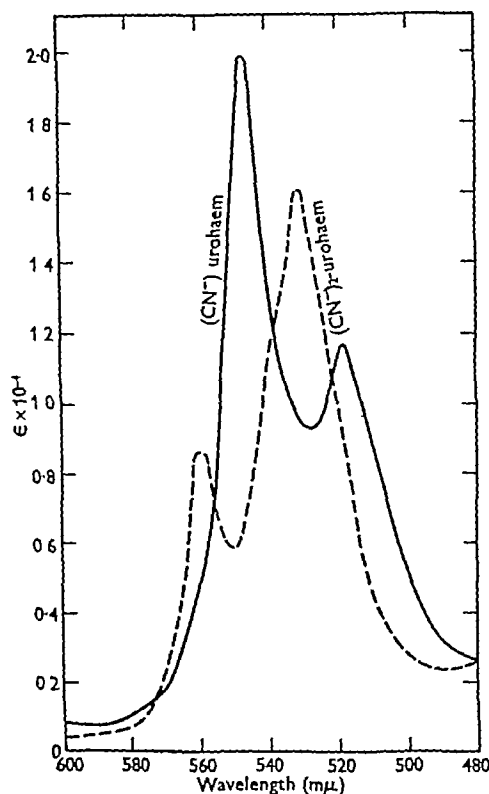


Fig 5 Absorption bands in the visible region of the spectrum of (CN^-) urohaem ($\text{Haem.KCN} = 1.36$) and $(\text{CN}^-)_2$ urohaem, containing excess KCN. Urohaem $= 0.55 \times 10^{-4} \text{ M}$ for first compound and $0.661 \times 10^{-4} \text{ M}$ for second compound, $l = 0.25 \text{ cm}$. The γ bands of these compounds are shown in Fig 7.

very similar to those of mono- and di-cyan urohaem. (CH_3NC) urohaem has its α , β and γ absorption bands at 547, 516 and 410 $\text{m}\mu$, the values of $\epsilon \times 10^{-4}$ being 1.66, 1.19 and 17.5, respectively (Figs 6 and 7).

$(\text{CH}_3\text{NC})_2$ Urohaem has α and β bands at 554 and 524 $\text{m}\mu$, the values of $\epsilon \times 10^{-4}$ being 1.1 and 1.55, respectively. The γ band of this compound, which lies at 422 $\text{m}\mu$, is of interest in that the value of $\epsilon \times 10^{-4}$ is about 26.8 (certainly above 25.6) (Figs 6 and 7). The only other metalloporphyrin known which possesses such a high γ band is turacin, a

naturally occurring copper uroporphyrin. In turacin the γ band is at 399 $\text{m}\mu$, and $\epsilon \times 10^{-4}$ is 26.

The reaction between protohaem and 10^{-4} M CH_3NC in alcoholic solution with cysteine buffer as reducing agent was described by Warburg, Negelein & Christian (1929) in the course of their investigations on the compound formed between methyl isocyanide and haemoglobin and on the effects of methyl isocyanide on the photochemical dissociation of CO haemoglobin. These authors give the absolute absorption spectrum of methyl carbylamine-haem of which only the γ band resembles that of $(\text{CH}_3\text{NC})_2$ -urohaem as shown in Fig 7. The mono carbylamine haem compound was not described by these authors.

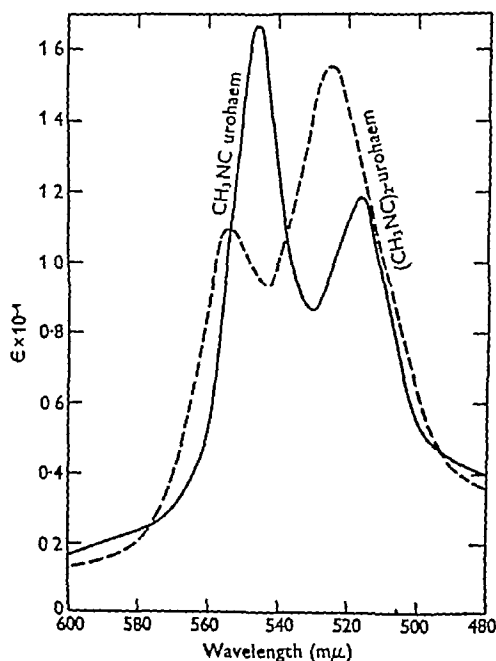


Fig 6 Absorption bands in the visible region of the spectrum of CH_3NC -urohaem (urohaem $\text{CH}_3\text{NC} = 1.1$) and $(\text{CH}_3\text{NC})_2$ urohaem (urohaem $\text{CH}_3\text{NC} = 1.150$). Urohaem $= 0.834 \times 10^{-4} \text{ M}$, $l = 0.25 \text{ cm}$.

Reactions of carbon monoxide with mono and di carbylamine haems. With (CH_3NC) protohaem, as in the case of (CN^-) protohaem, CO displaces the single molecule of CH_3NC from the haem Fe, and ordinary CO haem is obtained. When CH_3NC was added to an ethanolic solution of CO-protohaem so that the molecular ratio of CH_3NC to haem was 3:1, the α and β bands became more diffuse and a γ -band appeared at 422.5 $\text{m}\mu$ in addition to that of CO-haem (410 $\text{m}\mu$). This new band, which lay in a position intermediate between those of CO haem and $(\text{CH}_3\text{NC})_2$ haem, was most probably due to a CO-carbylamine haem compound, analogous to CO- (CN^-) haem. The same result is obtained if CO is

passed through a solution of $(\text{CH}_3\text{NC})_2$ haem containing CH_3NC and haem in the same 3:1 ratio. If more CH_3NC is present, e.g. 15 mol/mol of haem, the CO compound does not appear to be formed.

Reactions of methyl cyanide with haem. Whereas a commercial sample of methyl cyanide (CH_3CN)

Reactions of methyl isocyanide and methyl cyanide with the trivalent iron compounds, haematin and methaemoglobin.

Haematin. KCN reacts readily with haematin causing the colour to change from greenish brown to scarlet, and the narrow absorption band at about $615\text{ m}\mu$ of alkaline protohaematin to be replaced by a broad, somewhat diffuse band in the green region of the spectrum at $545\text{ m}\mu$. According to Hogness, Zscheile, Sidwell & Barron (1937) cyan haematin contains 2 mol of cyanide/mol of haematin. If, however, CH_3NC or CH_3CN is added to protohaematin in ethanolic solution, there is no change in the haematin spectrum, an indication that neither of these compounds reacts with haematin. Similar negative results were obtained with urohaematin.

Methaemoglobin. KCN also combines with acid methaemoglobin, altering the colour of the solution from reddish brown to bright red and causing the absorption band at $630\text{ m}\mu$ to be replaced by a broad band the centre of which lies at about $540\text{ m}\mu$. Neither methyl isocyanide nor methyl cyanide causes any change in the colour or spectrum of methaemoglobin, and it may therefore be assumed that these compounds do not combine with it.

DISCUSSION

Haemochromogens have hitherto been defined as compounds of haem with 2 mol of a nitrogenous base. Potassium cyanide and methyl isocyanide, however, differ from all other nitrogenous substances investigated so far, in that each can form with haem two distinct compounds containing 1 and 2 mol of the substance per molecule of haem.

In his study of the two cyanide haem compounds, Hill (1929) found that on the addition of CO to (CN^-) haem, ordinary CO haem was formed and not a CO (CN^-) haem compound, the single cyanide ion being replaced by CO. He stated, however, that 'the existence of a compound such as CO cyan-haemochromogen is not excluded, but so far it has not been detected'. It is now shown that there is ample spectroscopic evidence for the existence of a definite CO (CN^-) haem. The conditions required for its formation show that in the presence of excess cyanide, i.e. when $(\text{CN}^-)_2$ haem is formed, one of the CN^- groups of this compound can be replaced by CO giving CO (CN^-) -haem. Apparently in (CN^-) haem, CO can replace only the (CN^-) group and not the water co-ordinated with the iron atom, as shown in the scheme on p. 444.

The CO (CN^-) haem is of special interest since it shows that two important inhibitors of respiration and of reactions catalysed by certain haematin compounds may, under certain conditions, co-ordinate simultaneously with the same iron atom of haem.

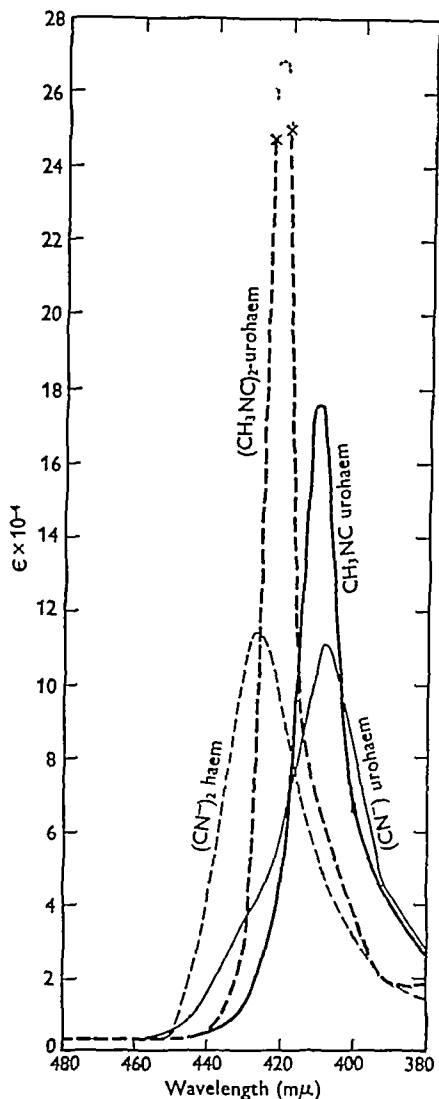


Fig. 7. Absorption spectra showing γ bands of $(\text{CN}^-)_2$ urohaem, (CN^-) urohaem (solutions as in Fig. 5), $(\text{CH}_3\text{NC})_2$ urohaem and CH_3NC -urohaem (solutions as in Fig. 6) $l=0.25\text{ cm}$.

reacted with urohaem giving a 4 banded spectrum corresponding to a mixture of the mono- and di-cyan haem types of spectra, a pure specimen (b.p. 81°) which had been treated with $\text{N}_2\text{H}_4\text{SO}_4$ to hydrolyse any isocyanide present, followed by N-NaOH to remove any free HCN, failed to react with urohaem in any proportions in both aqueous and ethanolic solutions. The importance of this failure to react with haem will be discussed later.

A comparative study of the properties of the two cyanide protohaem compounds and ordinary haemochromogens (e.g. globin or pyridine haemochromogen) shows that (1) (CN^-) haem has an absorption spectrum of the same pattern as that of a haemochromogen but, unlike the latter, it contains only 1 mol of nitrogenous base/atom of Fe, its absorption bands both in the visible and violet regions of the spectrum are considerably lower than those of a haemochromogen, and it does not form a CO compound of the CO haemochromogen type. Unfortunately, its magnetic susceptibility has not yet been determined, for it would be very interesting to know whether or not the compound is diamagnetic as suggested by its absorption spectrum. (2) $(\text{CN}^-)_2$ haem resembles a haemochromogen in its composition (2 mol of nitrogenous substance per atom of Fe), in the zero magnetic moment of the Fe atom (Pauling & Coryell, 1936) and in forming a CO (CN^-) -haem compound analogous to a CO haemochromogen, but it differs from a haemochromogen in several ways. The patterns of the absorption spectra of $(\text{CN}^-)_2$ -haem and its CO derivatives are entirely different from those of a haemochromogen and CO haemochromogen respectively and, in addition, the densities of the absorption bands of $(\text{CN}^-)_2$ -haem are only about half of those of a typical haemochromogen.

Methyl isocyanide has now been shown to give two compounds with haem analogous to those given by KCN. Since methyl isocyanide (CH_3NC) is only able to combine with the haem Fe by means of its terminal carbon atom, it may be assumed that in both mono- and di-cyan haem the cyanide ion is also linked by its carbon atom. Additional evidence in favour of this view is provided by the inability of methyl cyanide (CH_3CN) to react with haem either in the manner of KCN or as a simple haemochromogen forming base in spite of its terminal nitrogen atom. These results corroborate the views of Pauling & Coryell (1936) and Wyman (1948) that 'in the dicyanide compound it is probably the carbon atoms of the cyanide ions which are linked with the iron'. It is this mode of linkage which is probably responsible for the fundamental differences between the cyanide and carbylamine haem compounds on the one hand and the true haemochromogens on the other. In the latter type of compound the nitrogenous base is always linked to the haem Fe through the nitrogen atom.

It is of interest to note that methyl isocyanide reacts with the bivalent iron of haem and haemoglobin but not with the trivalent iron of haematin and methaemoglobin. In this respect methyl iso-

cyanide resembles CO, with which it can compete for ferrous haem and haemoglobin.

The structure of dicyan haem is of particular interest since the pattern of its absorption spectrum bears a strong resemblance to a certain haem derivative which was first described by Dhéré & Vegezzi (1916) and which will be discussed in the next paper.

SUMMARY

1 As was previously shown, potassium cyanide gives rise to two distinct compounds with haem: monocyan haem ($1 \text{ CN}^-/\text{Fe}$) and dicyan haem ($2 \text{ CN}^-/\text{Fe}$).

2 The absorption curves of these compounds have been determined in the visible as well as the violet regions of the spectrum and the marked differences in their pattern are discussed in relation to the spectra of other haematin derivatives.

3 Methyl isocyanide (CH_3NC) combines with haems to give mono- and di-carbylamine haem compounds having the same spectroscopic properties as mono- and di-cyan haems, whereas methyl cyanide (CH_3CN) does not combine with haems. These results show that the cyanide ions in mono- and di-cyan haems are attached to the haem iron through their carbon atoms. This mode of attachment accounts for the difference between the absorption spectra of $(\text{CN}^-)_2$ haem or $(\text{CH}_3\text{NC})_2$ haem and those of ordinary haemochromogen compounds such as pyridine haemochromogen.

4 It is only when one cyanide ion or one molecule of methyl isocyanide is attached to the haem iron through its carbon atom that the absorption spectrum of the resulting compound resembles that of an ordinary haemochromogen.

5 Methyl isocyanide, like carbon monoxide, combines only with haem and haemoglobin and does not react with the ferric compounds haematin and methaemoglobin, both of which form characteristic compounds with cyanide ion.

6 Methyl cyanide was found not to react with any of the haematin compounds examined.

7 The existence of a CO (CN^-) haem compound in which iron is simultaneously co-ordinated with cyanide and carbon monoxide has been demonstrated, and an analogous CO (CH_3NC) haem compound is also described.

I wish to thank Dr R. Hill, F.R.S., and Dr E. F. Hartree for their valuable suggestions, Prof C. H. Gray for putting me in contact with a case of congenital porphyria without whose ready co-operation much of this work could not have been carried out, and Mr C. L. Tsou for the preparation of a sample of methyl isocyanide. Thanks are also due to the Medical Research Council for a personal grant.

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On the Properties and Nature of Dihydroxyl-haem

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(Received 2 May 1949)

Dhéré & Vegezzi (1916), while working on the reduction of haematin under different conditions, described a compound obtained by reducing an ethanolic solution of haematin in strong alkali with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) in air. This compound, which they named 'alkaline haemochromogen', was red in colour and showed a characteristic absorption spectrum consisting of two bands, of which the β -band lying nearer the blue end of the spectrum was much stronger than the α -band. It was not until several years later, however, that the term 'haemochromogen' was defined by Anson & Mirsky (1925) as covering the compounds of reduced haematin or haem with different nitrogenous substances, and Hill (1926) showed conclusively that in a haemochromogen 2 mol of an organic base are combined with the one iron atom of haem. Of all the nitrogenous compounds examined only potassium cyanide and methyl isocyanide (methyl carbylamine) were found to form two distinct compounds with haem: monocyan-haem and dicyan haem, which contain 1 and 2 mol of cyanide per molecule of haem respectively, and mono- and di carbylamine haem (see Keilin, 1949).

The object of this investigation is to elucidate the nature of the compound described by Dhéré & Vegezzi as 'alkaline haemochromogen' and to compare it with haem, monocyan-haem, dicyan haem and haemochromogens. It is proposed to describe the 'alkaline haemochromogen' of Dhéré & Vegezzi under the name of dihydroxyl haem, with a prefix to the haem denoting the type of porphyrin used, e.g. dihydroxyl protohaem and dihydroxyl urohaem.

MATERIAL AND METHODS

Protohaemin This was prepared by the method of Schallfejeff (1885) from ox or horse blood.

Haematohaemin Haematoporphyrin was prepared from protohaemin by Nencki's method (Nencki & Seiber, 1888) and the iron was introduced by treating the porphyrin with FeSO_4 in the presence of glacial acetic acid and sodium acetate.

Urohaemin Uroporphyrin I was isolated from the urine of a case of congenital porphyria. The porphyrin was esterified and the urohaemin prepared from the octamethyl ester according to Fischer & Orth (1934).

Spectroscopic observations For all qualitative spectroscopic examinations and for preliminary quantitative experiments a microspectroscope was used as previously described (Keilin, 1943). The recording of absorption spectra in the visible and violet regions was carried out with a Beckman photoelectric spectrophotometer. In all figures the molecular absorption coefficients (ϵ), as previously defined (Keilin, 1949), were plotted against wavelengths (in μ).

RESULTS

The reaction of protohaem with sodium hydroxide

A solution of protohaem, obtained by dissolving protohaemin in 0.1N NaOH or 1% (w/v) Na_2CO_3 (anhydrous) and reducing it with $\text{Na}_2\text{S}_2\text{O}_4$, shows two very diffuse absorption bands. The addition of ethanol to this solution affects this spectrum only in so far as it causes a very slight sharpening of the bands. If, however, the ethanolic solution of protohaematin contains a much higher concentration of NaOH, the solution becomes cherry coloured on reduction, and a characteristic two banded spectrum is seen in which the β band, with its centre at

558 $m\mu$, is much stronger than the α band which lies at 590 $m\mu$ (Fig 1) The γ band of this compound

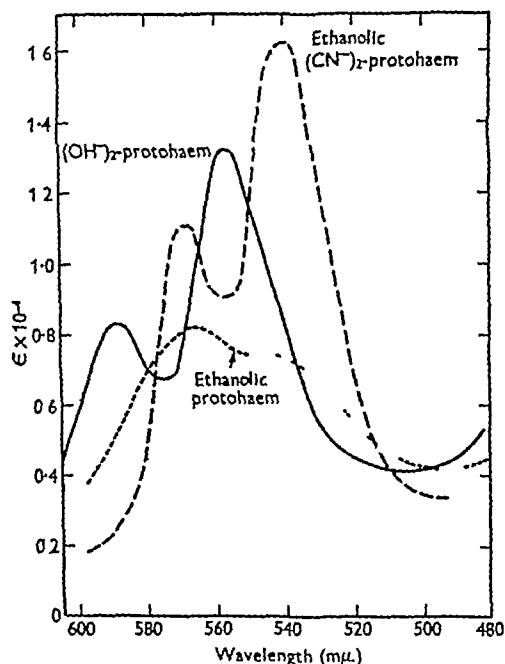


Fig 1 Absorption bands in the visible region of the spectrum of $(OH^-)_2$ protohaem, $(CN^-)_2$ protohaem containing 50% v/v ethanol (protohaem = 1.85×10^{-5} M) and ethanolic protohaem (Protohaem = 1.65×10^{-4} M, $l = 0.5$ cm)

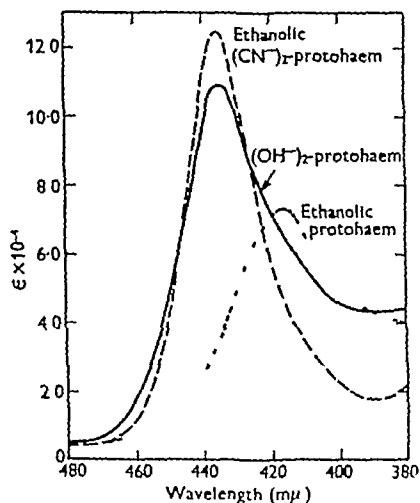


Fig 2 Absorption spectra showing the γ bands of $(OH^-)_2$ protohaem, ethanolic protohaem and $(CN^-)_2$ protohaem containing 50% v/v ethanol (Protohaem = 1.85×10^{-5} M, $l = 0.5$ cm)

responding band ($\lambda = 412$ $m\mu$, $\epsilon \times 10^{-4} = 7.2$) of ethanolic protohaem which, for the purposes of this paper, may be defined as a solution of haematin in a 50% (v/v) mixture of 0.1 N NaOH and ethanol, reduced with $Na_2S_2O_4$ (Fig 2) Dihydroxyl protohaem can also be obtained if the ethanol is replaced by acetone or glycol monomethyl ether. The effect of ethanol is therefore not specific, and the formation of this compound requires the presence of almost any water miscible solvent in addition to a high concentration of NaOH.

The optimum conditions for the formation of dihydroxyl protohaem were determined by varying the concentration of (a) ethanol or (b) NaOH, the concentrations of the other reagents remaining constant in both cases.

(a) *Experiments with varying concentrations of ethanol* For this purpose a series of tubes was set up each containing 1 ml of a 4.6×10^{-4} M solution of protohaematin in 1% (w/v) Na_2CO_3 and 1 ml of 10% (w/v) NaOH. To all except the first tube were added varying quantities of absolute ethanol. The solutions were made up to a constant volume of 4 ml with distilled water, reduced with dry $Na_2S_2O_4$ and examined spectroscopically.

The solutions containing 28.5% (v/v) or less of ethanol were opalescent, of a greenish brown colour and showed the spectrum of ordinary protohaem. When the concentration of ethanol reached 34% (v/v) the absorption spectrum of dihydroxyl protohaem was faintly seen, superimposed on that of haem. With increasing concentrations of ethanol, the absorption bands of dihydroxyl protohaem gradually replaced those of protohaem. The bands reached maximal intensity and the solution became clear and of a brownish pink colour only when it contained about 45% (v/v) ethanol. As a slightly higher concentration of ethanol did not affect the absorption spectrum of the compound, it was decided to add ethanol to 50% of the total volume of the solution in all subsequent experiments with dihydroxyl protohaem.

(b) *Experiments with varying concentrations of sodium hydroxide* In an attempt to determine whether the formation of the dihydroxyl protohaem was a function of the pH of the solution, Na_2CO_3 was used as the alkali, but even saturation of a solution of ethanolic protohaem with Na_2CO_3 failed to produce the characteristic spectrum. It was found that only strong alkali such as NaOH would give rise to this spectrum and the minimal concentration for its complete formation was determined spectrophotometrically as follows. A stock solution of protohaematin was prepared containing 12.35 mg protohaematin in 25 ml 1% (w/v) Na_2CO_3 and a series of 10 tubes was set up each containing 1 ml of the stock haematin solution and 3 ml absolute ethanol. To tubes 2-10 were added varying amounts of

($\lambda = 435$ $m\mu$, $\epsilon \times 10^{-4} = 11$) is much stronger and lies nearer the red end of the spectrum than the cor

NaOH ranging from 0.5 ml *N*-NaOH to 2 ml 12*N* NaOH and the volumes of all solutions were adjusted to 6 ml with distilled water. The solutions were reduced with solid $\text{Na}_2\text{S}_2\text{O}_4$ and allowed to stand for about 30 min to allow full reduction to take place, especially in the more alkaline solutions. The maximum intensities of the absorption bands were measured for each solution, and it was found that a concentration of 1.33*N* NaOH provided the optimum conditions for the formation of dihydroxyl protohaem.

The choice of solvent used for the formation of dihydroxyl protohaem. In the reaction described above ethanol was used as originally specified by Dhéré & Vegezzi (1916). The reaction, as has already been mentioned, is also given with acetone or glycol monomethyl ether, both of which are water miscible solvents. But dioxan used in conjunction with NaOH, though freely miscible with water, failed to give dihydroxyl protohaem. When dioxan was added to a solution of haematin either in 1% (w/v) Na_2CO_3 or in 0.1*N*-NaOH in the presence of $\text{Na}_2\text{S}_2\text{O}_4$, the solution became a clear cherry red and its two banded absorption spectrum closely resembled that of caffeine haem (Keilin, 1943). However, dioxan greatly sharpened the absorption spectrum of CO protohaem both in aqueous and ethanolic solution, shifting the absorption bands a little towards the blue end of the spectrum, in contrast to the action of caffeine on CO protohaem.

Reactions of haems other than protohaem with sodium hydroxide

Whereas protohaem requires ethanol or a similar solvent for the formation of dihydroxyl protohaem, other haems such as deuterio-, haemato- and urohaem form their corresponding dihydroxyl compounds without the addition of any organic solvent (Fig. 3). None of these haems, however, would give dihydroxyl compounds with Na_2CO_3 instead of NaOH. The formation of dihydroxyl haematohaem and dihydroxyl urohaem with increasing concentrations of NaOH were followed spectrophotometrically as described for dihydroxyl protohaem. The minimum concentration of NaOH required for the formation of dihydroxyl haem compounds was found to be 1.33*N* for protohaem in the presence of 50% ethanol and only 1.0*N* for haemato- and urohaem in the complete absence of ethanol. The presence of ethanol, nevertheless, slightly sharpens and shifts the absorption bands of dihydroxyl haematohaem towards the red end of the spectrum. It affects similarly, but to a lesser degree, those of dihydroxyl urohaem.

Stoichiometric relationships

In order to determine the number of hydroxyl groups combined with each molecule of haem to give

the hydroxyl compound, the haem was titrated with NaOH using the double wedge trough method described by Keilin & Hartree (1946). Haematohaem was selected for this experiment, since it does not require ethanol for the formation of its hydroxyl compound and is more stable to reoxidation by air than urohaem.

3 ml of a 6.12×10^{-4} *M* solution of haematohaematin in 1% Na_2CO_3 were diluted with 1% Na_2CO_3 to 25 ml for one compartment of the double wedge trough, and with 1.5*M* NaOH for the other compartment, so that the haematin concentration was 7.35×10^{-5} *M* in each compartment of the

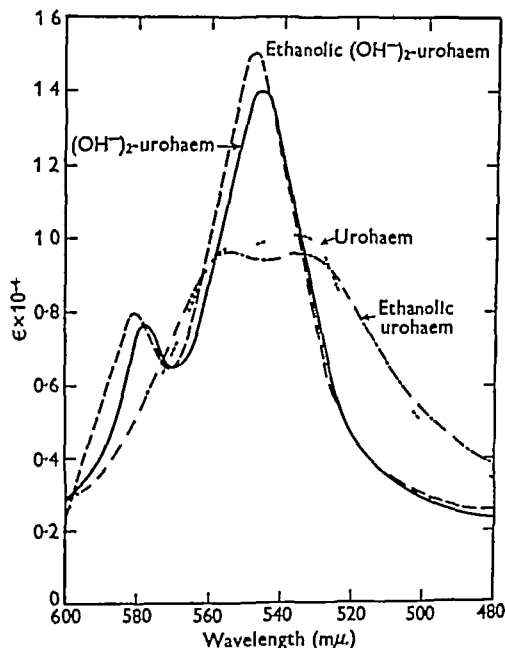


Fig. 3 Absorption bands in the visible region of the spectrum of $(\text{OH}^-)_2$ urohaem, urohaem, ethanolic urohaem and ethanolic $(\text{OH}^-)_2$ urohaem. The two latter solutions contain 50% v/v ethanol. (Urohaem = 1.263×10^{-4} *M*, $l = 0.5$ cm.)

trough. The volume (*v*) of 6.12×10^{-4} *M* haematohaematin for the required optical depth in the cup was calculated as follows: $v = \pi r^2 (c_1 l / c_2)$, where c_1 and c_2 = haematohaematin concentrations in trough and cup, respectively, l = light path in trough (2.1 cm) and r = radius of cup. All the solutions were reduced with $\text{Na}_2\text{S}_2\text{O}_4$ and the haematohaem in the cup was matched against the broad end of the haematohaem compartment of the trough which was at zero position, the light intensities of the two spectra having been carefully equalized. The haem in the cup was then titrated with *N* NaOH and the spectrum of the solution was matched, after each addition of NaOH, against the optical mixture of haematohaem and its hydroxyl compound in the two compartments of the trough. The concentrations

Table 1 The relation between the sodium hydroxide concentration and percentage formation of dihydroxyl haematohaem as determined experimentally and as calculated from the results by the method of least squares

Total NaOH added (ml)	NaOH concentration in solution		Percentage formation of compound (x)	Percentage free haem (a - x)	log {x/(a - x)}	
	M	log M			Determined experimentally	Calculated
0.05	0.238	-0.6231	16	84	-0.7201	-0.718
0.10	0.384	-0.4157	36	64	-0.2495	-0.286
0.25	0.612	-0.2132	56	44	+0.0864	+0.135
0.35	0.888	-0.1624	65	35	+0.2669	+0.240
0.45	0.740	-0.1308	62	38	+0.2253	+0.306
0.55	0.775	-0.1107	70	30	+0.3674	+0.348
0.70	0.814	-0.0894	72	28	+0.4099	+0.392
0.80	0.825	-0.0835	73	27	+0.4314	+0.409

of haematohaematin in the cup and trough were specially selected so that the spectrum of the haematohaem was clearly visible, while that of the fully formed hydroxyl compound in the trough

then from the law of mass action

$$\frac{[\text{Haem}(\text{OH}^-)_n]}{[\text{Haem}][\text{OH}^-]^n} = K$$

If x represents the amount of the hydroxyl compound formed and a represents the total haem present, then

$$\frac{x}{(a-x)[\text{OH}^-]^n} = K,$$

whence $\log \{x/(a-x)\} = n \log [\text{OH}^-] - \log K$

By applying the method of least squares to the values obtained for $\log \{x/(a-x)\}$ and $\log [\text{OH}^-]$, it was found that $n = 2.08$ and $\log [\text{OH}^-] = -0.278$ at 50% formation of the hydroxyl compound while $k = 0.264$, since under these conditions $\log K = n \log [\text{OH}^-]$ (This value for k does not take into account the very slight depression of $[\text{OH}^-]$ due to the presence of $\text{Na}_2\text{S}_2\text{O}_4$, nor the activity coefficient of NaOH). On substituting these values for n and K in the above equation, the theoretical values of $\log \{x/(a-x)\}$ for different concentrations of NaOH can be calculated. These calculated values, together with those obtained experimentally, are shown in Fig 4. This experiment shows conclusively that in the hydroxyl haematohaem compound the haem Fe combines with two hydroxyl groups, and since the corresponding compounds obtained with other haems have similar properties, all these compounds may be described under the name of dihydroxyl haems.

Reactions of dihydroxyl haems with carbon monoxide

When a current of CO is passed through a solution of dihydroxyl protohaem, the latter assumes a bright cherry red colour and its absorption bands are shifted to the following positions α , 563, β , 532 and γ , 410 $\text{m}\mu$. This spectrum corresponds to that of a solution of ethanolic CO protohaem (Fig 5). Similarly, the CO derivatives of dihydroxyl haematohaem and dihydroxyl urohaem in aqueous solution are indistinguishable from their respective aqueous solutions of CO haems.

At this point it may be interesting to recall Dhéré & Vegezzi's (1916) experiments on the

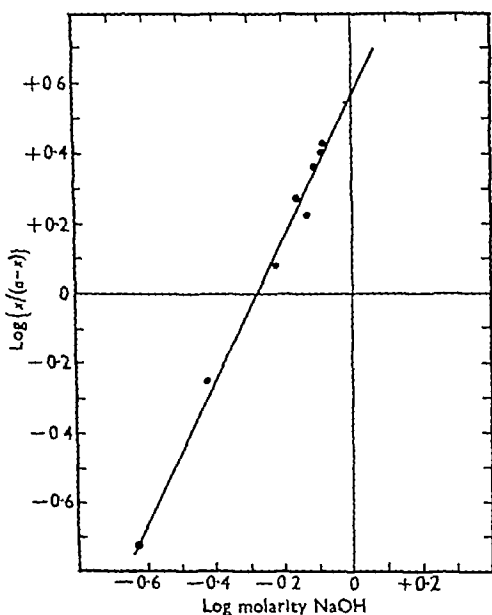


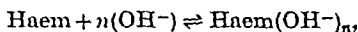
Fig 4 Relationship between log molarity of NaOH and $\log \{x/(a-x)\}$, where x = percentage formation of $(\text{OH}^-)_2$ haematohaem and $a-x$ = percentage of free haematohaem. Experimental results are represented by dots. Results calculated from the equation

$$\log \{x/(a-x)\} = n \log [\text{OH}^-] - \log K,$$

where $n = 2.08$ and $K = 0.264$, are represented by the straight line.

(containing excess NaOH) was not too intense to prevent matching with the solution in the cup. It was thus possible to determine the percentage formation of the hydroxyl compound at different concentrations of NaOH and the results are given in Table 1.

If the reaction is represented by the following equation



reduction of acid and alkaline ethanolic solutions of protohaematin in open and sealed tubes. They invariably found that in sealed tubes the absorption spectra of the solutions were very different from those in open tubes and, although the latter contained an excess of reducer, they believed at first that complete reduction could only be obtained in sealed tubes. However, later Dhéré (1927) recognized that the difference was not due to the more complete reduction of the solutions in sealed tubes but to the formation within them of CO protohaem, the CO having been produced by incomplete combustion of ethanol during the process of sealing the tubes.

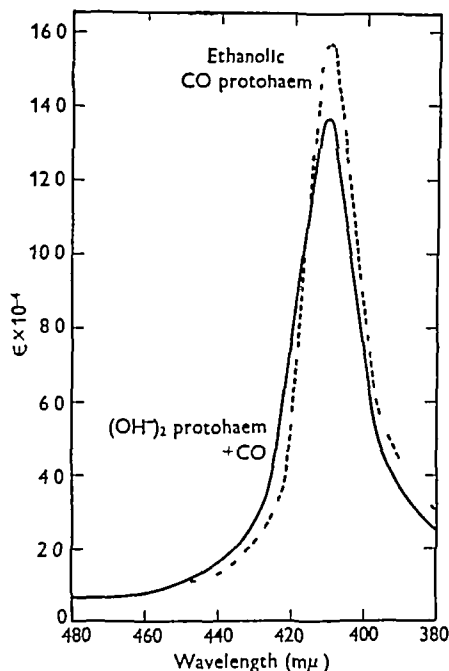


Fig 5 Absorption spectra showing γ bands of $(\text{OH})_2$ protohaem + CO and of CO protohaem containing 50% v/v ethanol. (Protohaem = 1.85×10^{-5} M, $l = 0.5$ cm)

The magnetic susceptibility of dihydroxyl urohaem

The method of Gouy (1889) was adapted for use with an 'Alcomax' permanent magnet giving a uniform field of 10,600 gauss within a gap of 6 mm. The magnet was mounted on a jack so that it could be moved vertically with precision and without any lateral displacement of the magnet. The magnet and jack were enclosed in a glass case and kept at constant temperature (20.5°). A narrow glass tube (10 cm long, 2 mm internal diam and 0.3 ml capacity), fitted with glass suspending hooks at the open end and closed with a rubber cap, was hung on a platinum wire from the pan of the balance and weighed with its lower end between the pole pieces of the magnet. The magnet was then lowered about 25 cm by means of the jack, and the tube, now in a negligible magnetic field, was weighed again. The increase in weight, Δw , was then similarly determined (a) with the tube full of distilled water, (b) with the tube filled with the haem solution

under investigation, and (c) with the tube filled with the solvent (i.e. all the constituents except the haem). These changes in weight were each corrected for the change in weight of the empty tube Δw , and are represented as $\Delta w_{\text{H}_2\text{O}}$, Δw_{soln} and Δw_{solr} respectively.

The mass susceptibility χ of the solution was calculated according to the following formula, derived from the equation given by Trew & Watkins (1933)

$$10^6 \chi_{\text{soln}} = \left(10^6 \chi_{\text{H}_2\text{O}} - \frac{K_{\text{air}}}{d_{\text{H}_2\text{O}}} \right) \frac{\Delta w_{\text{soln}}}{\Delta w_{\text{H}_2\text{O}}} \frac{d_{\text{H}_2\text{O}}}{d_{\text{soln}}} + \frac{K_{\text{air}}}{d_{\text{soln}}},$$

where $\chi_{\text{H}_2\text{O}}$, the mass susceptibility of water = -0.72×10^{-6} , K_{air} , the volume susceptibility of air = $+0.0294 \times 10^{-6}$, and d = specific gravity.

The value of $10^6 \chi_{\text{solr}}$ was similarly calculated and the mass susceptibility of the solute (haem) was obtained from

$$\chi_{\text{soln}} = c \chi_{\text{solute}} + (1 - c) \chi_{\text{solr}}$$

where c = wt of dissolved haem/wt of solution.

The magnetic moment μ_B (in Bohr magnetons) is then given by

$$\mu_B = 2.84 \sqrt{(\chi M T)},$$

where χ = mass susceptibility of haem compound, M = mol. wt of haem, T = absolute temperature.

Urohaem was selected for these experiments instead of protohaem in view of its greater solubility and the ease in bringing the reaction to completion. The magnetic susceptibilities of protohaematin, dicyan protohaem and dicyan urohaem were also determined by the same method. This was done in order (1) to test the accuracy of the procedure, (2) to test the suitability of the urohaem preparation for such measurements, and (3) to provide the data necessary for comparison with those obtained for dihydroxyl urohaem.

The solutions were made up as follows.

Protohaematin. Protohaemin (50 mg) was dissolved in 5 ml 0.2N NaOH containing about 30% sucrose (Pauling & Coryell, 1936).

Dicyan protohaem. Protohaemin (60 mg) was dissolved in about 6 ml of the above sucrose NaOH solution. KCN (300 mg) and $\text{Na}_2\text{S}_2\text{O}_4$ (100 mg) were then added and the volume of the solution was made up to 10 ml with sucrose NaOH. (This order was adopted to avoid the gelatinous precipitate which tended to form if the solid haem was added last.)

Dicyan urohaem. Urohaemin (20 mg), KCN (30 mg) and $\text{Na}_2\text{S}_2\text{O}_4$ (20 mg) were dissolved in that order in 1 ml 1% Na_2CO_3 contained in a small, stoppered, graduated bottle with a narrow neck to minimize the air space above the fluid. The solution was transferred by means of a Pasteur pipette to the magnetic susceptibility tube which was completely filled and closed with the rubber cap. These precautions to exclude contact with air were necessary on account of the extreme ease with which the reduced urohaem compounds are oxidized in air.

Dihydroxyl urohaem. Urohaemin (20.7 mg) and $\text{Na}_2\text{S}_2\text{O}_4$ (20.3 mg) were dissolved in 1 ml. N NaOH, observing the precautions described above.

The results of these experiments, which were carried out at 20.5° and which are summarized in Table 2, show that the Fe in dihydroxyl urohaem has a much higher magnetic moment than the Fe in dicyan urohaem. The values obtained for protohaematin and dicyan protohaem are in agreement with those given by Pauling & Coryell (1936) for

Table 2 The values for Δw and specific gravity (d) of water, solvent and solution, and the molar susceptibility χ_m and magnetic moment μ_B (in Bohr magnetons) of protohaematin, dicyan protohaem, dicyan urohaem and dihydroxyl urohaem

(Approx 30% sucrose was used only in the case of protohaematin and dicyan protohaem)

Compound	Solvent			Δw_{H_2O}	$d_{H_2O}^{20.5}$	Δw_{soln}	$d_{soln}^{20.5}$	Δw_{soln}	$d_{soln}^{20.5}$	$10^3 \chi_m$	μ_B
	$Na_2S_2O_4$ (mg/ml)	KCN (mg/ml)	Alkali and sucrose								
Protohaematin	—	—	30% sucrose, 0.2 N NaOH	4.142	0.998	4.2498	1.1193	3.048	1.1223	13,830	5.722
Dicyan protohaem	10	30	30% sucrose, 0.2 N NaOH	4.022	0.998	4.0724	1.1415	4.137	1.1455	0	0
Dicyan urohaem	20	50	1% Na_2CO_3	2.533	0.998	2.740	1.0412	2.631	1.0445	909	1.46
Dihydroxyl urohaem	20.1	—	N NaOH	2.44	0.998	2.70	1.056	1.673	1.0645	13,585	5.672

both compounds and by Haurowitz & Kittel (1933) for protohaematin. The relatively high values obtained for both the urohaem compounds are probably due to some degree of oxidation that occurred in spite of the precautions taken to avoid it.

DISCUSSION

The solubility of a haemin derivative depends very largely on that of its parent porphyrin and the porphyrins differ greatly in the nature of their side chains. Carboxyl groups in general confer solubility on the molecule so that uroporphyrin, with its eight carboxyl groups, is the most soluble of the porphyrins. Haematoporphyrin, with $-\text{CH}(\text{OH})\text{CH}_3$ groups in positions 2 and 4, is more soluble than proto-porphyrin which has vinyl groups ($-\text{CH}=\text{CH}_2$) in these positions. Haemin derivatives also differ in solubility according to the valency of their iron. Thus the ferrous compounds such as haem and haemochromogen are less soluble than their ferric forms, haematin and parahaematin. It is also interesting to note that CO haem is more soluble than free haem.

It is well known that the solution or dispersion of molecular aggregates of a pigment is accompanied by an intensification of its absorption bands and by a more or less marked shift of these bands towards the blue end of the spectrum. In fact, the addition of ethanol intensifies the absorption bands of haems, and this intensification, which varies inversely with the solubility of the haem in dilute aqueous alkali, is therefore more marked for proto- than for haematohaem and is very feeble in the case of urohaem.

The conditions required for the formation of dihydroxyl haem compounds are (a) a high concentration of alkali (1–1.33N NaOH), and (b) a certain degree of solubility of the haem. In the case of protohaem, which is relatively insoluble, the required degree of solubility can only be obtained by the addition of an appreciable amount of ethanol (up to 50%) or of another water miscible solvent

such as acetone or glycol monomethyl ether. On the other hand, ethanol is not required for the formation of dihydroxyl-urohaem and, when added, hardly

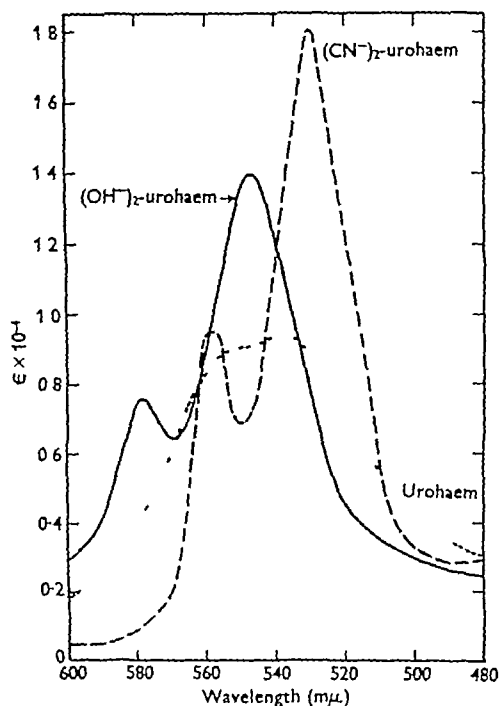


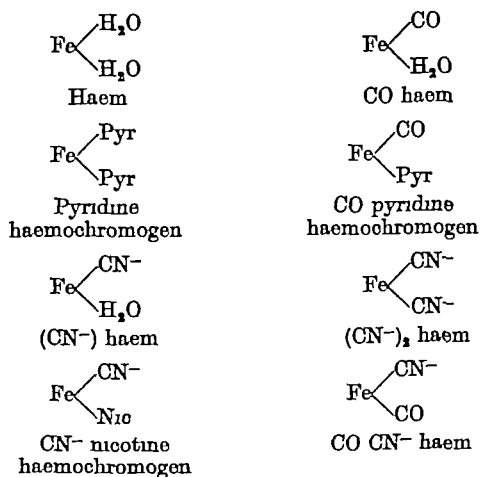
Fig. 6 Absorption bands in the visible region of the spectrum of $(\text{OH}^-)_2$ urohaem, urohaem (urohaem = 1.263×10^{-4} M, $l = 0.5$ cm) and $(\text{CN}^-)_2$ urohaem (urohaem = 6.61×10^{-5} M, $l = 0.25$ cm).

affects the spectrum, as urohaem itself is very soluble in very dilute alkali. In this respect haematohaem occupies an intermediate position between proto- and urohaem. Although dihydroxyl-haematohaem, with its characteristic absorption spectrum, may be formed in the complete absence of ethanol, the addition of the latter intensifies its bands and

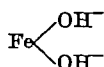
shifts them slightly towards the red end of the spectrum. This change is brought about by a simultaneous increase in the solubility of the haematohaem in presence of ethanol and a decrease in that of its dihydroxyl compound.

Information as to the nature of the hydroxyl-haem compound was obtained in two ways. (1) Directly, by titrating the haem with NaOH and determining its dissociation constant. This showed that two hydroxyl groups were combined with each atom of haem Fe (see Table 1 and Fig. 4). (2) Indirectly, by comparing its properties with those of other haem derivatives such as haem, caffeine haem, haemochromogens, monocyan- and dicyan haem and monocarbylamine- and dicarbylamine haem. The absorption spectra of dihydroxyl-haems and the corresponding dicyan haems bear a striking resemblance to each other (Figs. 1 and 6). This resemblance, which can be seen in the visible and the violet regions of the spectrum, applies to the general pattern of the absorption spectrum as reflected in the relative intensities of the bands. The fact that the general spectral pattern common to dihydroxyl haems, dicyan-haems and dicarbylamine haems differs so markedly from those of all other haematin derivatives suggests that these compounds have similar structures.

Spectroscopic and potentiometric investigations (see Anson & Mirsky, 1925, Hill, 1929, Davies, 1940, Shack & Clark, 1947) suggest that the structures of different haem (ferrous) compounds can be represented as follows (in these schematic representations the lines joining the haem Fe to the reacting groups do not indicate the nature of the valency bonds, the four valencies directed towards the pyrrole nitrogen atoms are omitted).



By analogy with dicyan haem, the structure of dihydroxyl haem may therefore be represented as



The study of Dhéré & Vegezzi's (1916) compound, apart from its structural relationship with different haem derivatives, is important in two other respects. (1) Optical and magnetic studies on different haematin compounds have suggested the existence of a certain empirical relationship between the pattern of their absorption spectra and the type of iron bonding in each compound as determined by magnetic susceptibility measurements (Theorell, 1942, Hartree, 1947). In this respect the dihydroxyl urohaem presents a striking exception. Although the absorption spectra of dihydroxyl urohaem and dicyan urohaem are of the same pattern, the magnetic measurements show fundamental structural differences. While the Fe atom in dihydroxyl urohaem is linked by ionic bonds, with the result that the compound shows the high magnetic moment characteristic of free haems, the bonds in dicyan urohaem are essentially covalent and the low magnetic moment brings it into line with the diamagnetic haemochromogens. In view of the strict analogies existing between the corresponding compounds of different haems it is reasonable to assume that the results obtained for urohaem may be taken to apply to dihydroxyl compounds of other haems. (2) Strong alkalis, with or without ethanol, are often used in studying the reactions between haems and different nitrogenous substances. However, unless one is familiar with the absorption spectrum of dihydroxyl-haem and aware of its possible appearance during the course of the reaction, the main absorption band of this compound can easily be mistaken for that of a partly developed haemochromogen, since its strong β band can be seen where the α band of the haemochromogen is expected to appear. This aspect of the problem will be discussed more fully elsewhere.

SUMMARY

1 It is shown that Dhéré & Vegezzi's (1916) 'alkaline haemochromogen' is a compound in which the haem iron is combined with two hydroxyl groups and it is therefore named dihydroxyl haem.

2 For the full formation of dihydroxyl protohaem 1.33N NaOH and 50% (v/v) ethanol are required. Ethanol, however, can be replaced by other water miscible organic solvents such as acetone and glycol monomethyl ether, but not by dioxan.

3 In the case of haematohaem and urohaem the corresponding dihydroxyl compounds can be obtained in complete absence of an organic solvent, although the absorption spectrum of dihydroxyl haematohaem is reinforced by ethanol.

4 The dissociation constant K for dihydroxyl-haematohaem is 0.264 at 20°.

5 The absorption spectrum of dihydroxyl haem differs markedly from those of haem, caffeine haem,

haemochromogens and monocyane haem, but shows a striking resemblance to those of dicyane haem and dicarballyamine haem

6 The bearing of the similarity of the absorption spectra of dicarballyamine haem, dicyane haem and dihydroxyl haem on the structure of this compound is discussed

7 Dihydroxyl haem, unlike dicyane haem, is paramagnetic, and when treated with carbon monoxide gives CO haem

8 The knowledge of the absorption spectrum and other properties of the dihydroxyl haem compound is of special importance for the study of the reactions between haems and nitrogenous compounds

I wish to thank Dr R. Hill, F.R.S., for his valuable suggestions and interest in this work, and Dr E. F. Hartree, for advice regarding the magnetic susceptibility determinations. Thanks are also due to the Medical Research Council for a personal grant

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The Semi-micro Estimation of Lactose Alone and in the Presence of Other Sugars

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(Received 13 April 1949)

In the course of studies on the intermediary metabolism of the mammary gland, and in particular the investigation of lactose formation in incubated tissues, it became necessary to devise improved methods for estimating this sugar in the presence of glucose and glycogen. The methods hitherto available for the estimation of small amounts of lactose, alone or in the presence of other sugars, are laborious and unsatisfactory. Those normally used for estimating lactose in milk suffer from a lack of specificity which makes them valueless in any investigation where mixtures of sugars and related carbohydrates are likely to be found, while those designed for more general use rely on the complementary techniques of yeast fermentation, for removing fermentable sugars, and the use either of reduction methods or of a lactose fermenting yeast to give the lactose equivalent of the residual fermented solutions (Grant,

1935; Scott & West, 1936). The application of paper chromatography to the separation and characterization of sugars in mixtures (Partridge, 1946, 1948), and the adaptation of this method to the estimation of sugars (Flood, Hurst & Jones, 1947; Hawthorne, 1947), are advances of great value, but the techniques are time consuming and, for other reasons, frequently unsuited for routine work (see Westall 1948).

In the hope of obtaining a method which would be more convenient in practice and yet have greater specificity than those ultimately dependent on estimations of reducing power, the colour reaction given by lactose with methylamine in alkaline solution, first observed by Fearon (1942) and later used in qualitative studies for detecting lactosuria in pregnancy and the early puerperium (Harwood, 1946; Archer & Haram, 1948), has been developed into a quantitative method. Fearon found that the

reaction was not given by other sugars (except maltose), or by many other substances, including lactic and pyruvic acids, normally associated with carbohydrate metabolism. Since maltose can readily be removed by fermentation the reaction clearly offered the possibility of a specific estimation for lactose which might satisfy an urgent need in studies on the metabolism of the mammary gland.

EXPERIMENTAL

Reagents

Lactose Pure bacteriological (Kerfoot)

Methylamine hydrochloride Prepared from 40% methylamine in water (L. Light and Co., Ltd.) by neutralizing with HCl, evaporating the solution to dryness on a water bath, extracting with and recrystallizing from ethanol.

Zinc hydrosulfide Prepared from zinc acetate by the method of Letonoff (1934).

Estimation of lactose alone in aqueous solution

The solution (8 ml containing 4–16 mg lactose) is pipetted into a boiling tube ($6 \times 1\frac{1}{4}$ in) and treated with 6 drops (0.32 ml.) 10% (w/v) methylamine HCl solution. The tube is loosely stoppered with a glass cap and the mixture heated in a boiling water bath. After heating for 7 min and 5 sec., 0.4 ml of 4*N* NaOH are added, and 10 sec later the tube is removed from the bath and the contents mixed and allowed to cool in air. The red colour develops in the stoppered tube during the course of the next 2 min, and is measured in a Spekker photoelectric absorptiometer using a spectrum green filter (Ilford no. 604, 520 $m\mu$) in conjunction with heat-absorbing filters (Hilger H503). The solutions are read against water in the second cell and with the drum setting initially at unity.

To ensure maximum colour development the estimations are carried out with strict adherence to a time schedule. They may be done conveniently in an overlapping series, when 6 or 7 may be completed in 1 hr. The full time scheme is as follows for the first two members, A and B, of a series.

0 sec	Add 6 drops 10% methylamine-HCl to A
15 sec	Place A in boiling water bath and insert stopper
7 min 20 sec	Add 0.4 ml 4 <i>N</i> NaOH to A
7 min 30 sec	Remove A from bath
9 min 0 sec	Add 6 drops 10% methylamine HCl to B
9 min 15 sec	Place B in boiling water bath and insert stopper
9 min 30 sec	Filter A directly into absorptiometer cell (This is unnecessary in the case of simple aqueous solutions, but essential when salt solutions containing Ca^{++} or Mg^{++} are used, or when the estimations are on biological extracts)
10 min 45 sec	Measure A in absorptiometer
16 min 20 sec	Add 0.4 ml 4 <i>N</i> NaOH to B, and continue as outlined above

Under these conditions the calibration curve for standard lactose solutions is linear over the range 4–16 mg (0.05–0.2%), below this range the increase in the colour with

increase in concentration is less pronounced, and the method, although useful, is decidedly less accurate for solutions between 0.025 and 0.05% (Fig. 1).

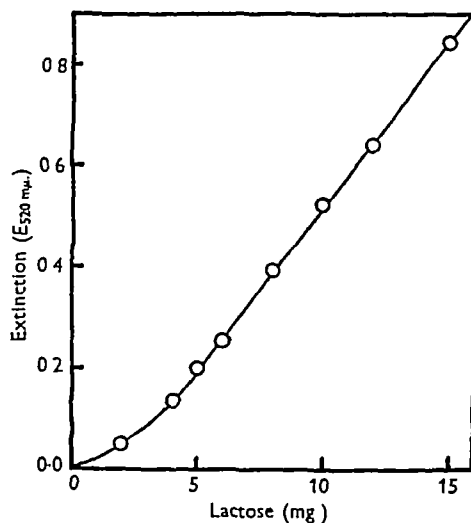


Fig. 1 Calibration curve for standard lactose solutions

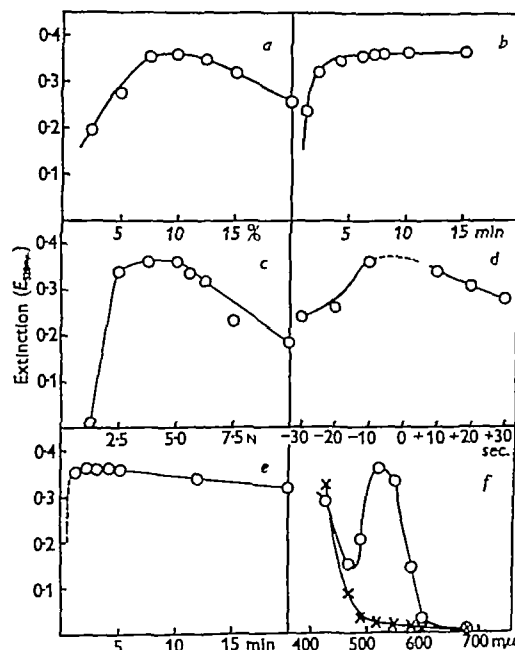


Fig. 2 Investigation of factors affecting the estimation of lactose (0.1% (w/v), cell 1 cm), (a) strength of methylamine HCl solution, (b) time of heating, (c) strength of NaOH, (d) time of addition of NaOH relative to removal from the water bath, (e) rate of development of colour after removal from the water bath, (f) absorption spectra of lactose compound \bigcirc — \bigcirc and glucose compound \times — \times

The results of an investigation into the optimum conditions of the reaction are given in Fig. 2. The effects of altering the strength of the methylamine HCl solution (a),

the time of heating (b), strength of NaOH (c), time of addition of NaOH relative to the time of removal from the water bath (d), and the time of estimating after removal

discussed in this paper 5N NaOH was used, from a reassessment of the method, however, it would seem preferable to use a slightly weaker solution and 4.4N NaOH is now

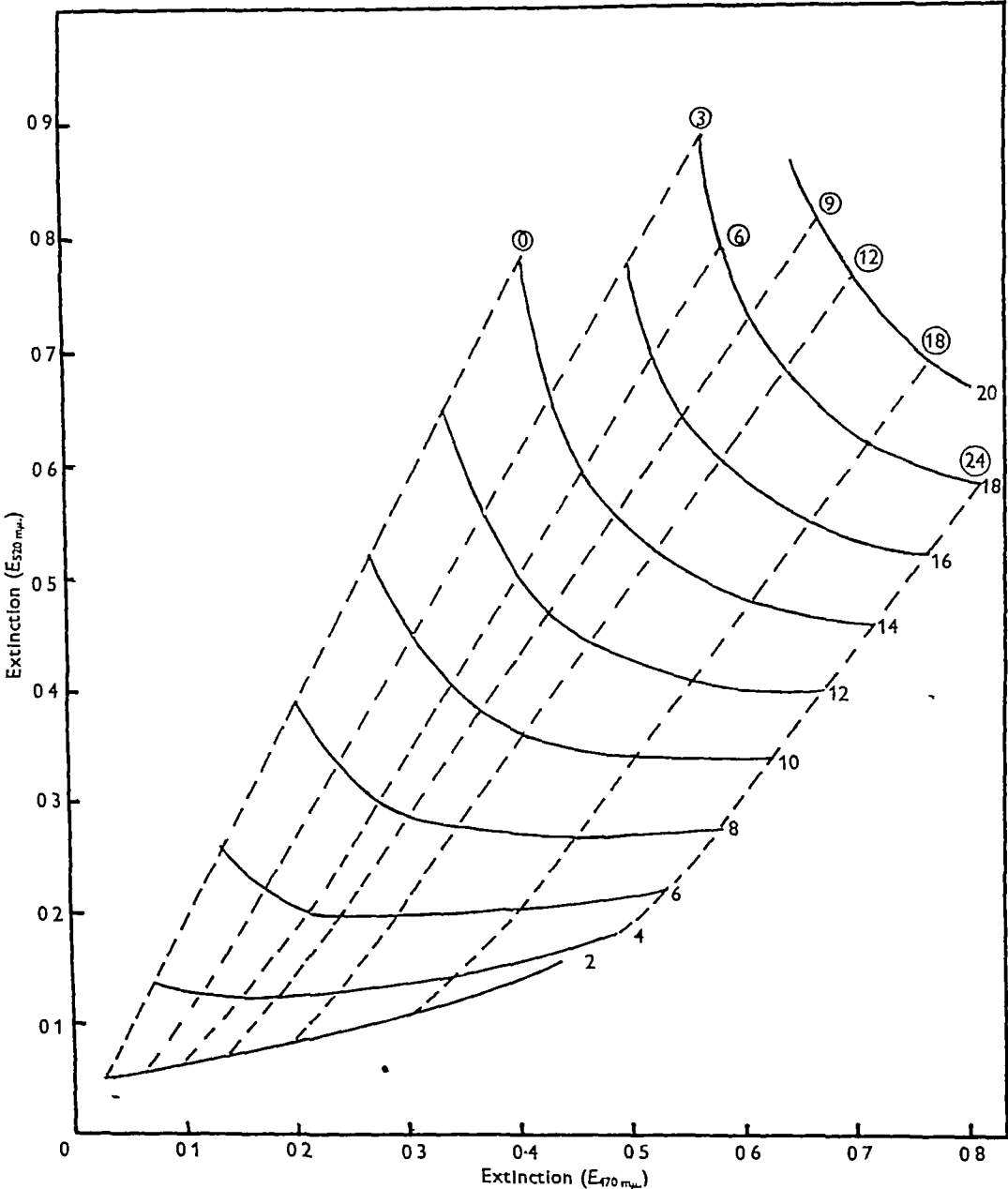


Fig 3 Composite graph used in estimating lactose in the presence of glucose —, curves for constant lactose concentration, equivalent (mg lactose) given in plain numbers, ---, curves for constant glucose concentration, equivalent (mg glucose) given in 'circled' numbers

from the water bath (e) are shown, together with the visible absorption spectrum of the lactose compound (f) The first, third and fourth of these possible variants are clearly critical and must be strictly controlled. In the further work to be

recommended The choice of the time for adding the NaOH relative to removing the tube from the water bath necessarily involves considerations of technical convenience, and although there are indications, both from experiment and

from an interpolation of partial curves (*d*), that the extinction values would be slightly higher if the removal coincided with the addition of NaOH, the small gain in sensitivity would be more than offset by the manipulative inconvenience

Estimation of lactose in the presence of glucose in aqueous solution

When estimated in the same way, glucose gives a yellow colour instead of red, and it is therefore possible to estimate lactose in the presence of glucose, without preliminary fermentation, by measuring the optical density at two wavelengths. The additional wavelength selected (470 m μ , Ilford no 602, used in conjunction with heat-absorbing filters, Hilger H 503) gives appreciable, but suboptimal, absorption for glucose alone (see absorption spectrum, Fig 2(f)), and coincides approximately with a point of relatively low absorption for lactose.

From the results given by a series of standard mixtures covering the range lactose 0–20 mg, glucose 0–24 mg, curves are drawn, by plotting the extinction at 520 m μ against the extinction at 470 m μ , relating these values to constant lactose concentrations measured in the presence of varying glucose concentrations (Fig 3). From these curves lactose values may be read directly for any pair of extinction values. The method introduces a source of subjective error in the need for interpolating values between the standard curves thus obtained. Interpolation is made on lines parallel to the transverse curves (broken lines in Fig 3) connecting points of the same glucose content on the different lactose curves. These transverse curves clearly allow the simultaneous determination of glucose in the solutions by a similar process of interpolation, this time along lines parallel to the standard lactose curves. The following examples may be demonstrated on Fig 3.

Example I Extinction 520 m μ = 0.200, extinction 470 m μ = 0.240. These readings give a point on the intersect of the 6 mg standard lactose curve and the 9 mg transverse glucose curve, and indicate a solution containing 6 mg lactose and 9 mg glucose.

Example II Extinction 520 m μ = 0.272, extinction 470 m μ = 0.238. These readings involve interpolation for both the lactose and the glucose values, and give the result, 7.2 mg lactose and 5 mg glucose.

Sensitivity of the method

For simple solutions of lactose the accuracy of the method in the most favourable range (0.05–0.2%) falls well within $\pm 5\%$. In the presence of glucose the accuracy varies with the relative amounts of the two sugars, and the direct application of the method is not recommended, except for qualitative work, for solutions containing less than 0.05% lactose, or for any solution containing more than 0.15% glucose. In such cases preliminary fermentation should be carried out and the lactose determined alone in the fermented solution. It is our experience that in the range suggested here lactose can be estimated with an accuracy of $\pm 5\%$, the glucose value is much less accurate (Table 1).

Estimation of lactose in the presence of glycogen

Solutions of glycogen give a strong opalescence when treated according to the standard method, and it is essential to remove glycogen before the estimation. This is done by precipitation with an equal volume of ethanol.

After standing for 5 min the solution is filtered and suitable samples of the ethanolic filtrate transferred to boiling tubes, the solutions are evaporated to dryness on a water bath, the residue dissolved as far as possible in 8 ml distilled water and the estimation carried out as in the standard method. If salts are present it is necessary to adjust the pH of the solutions to between 7.0 and 7.4 before drying, in order to prevent decomposition or charring of the sugar.

Table 1 *Recoveries of lactose from lactose-glucose solutions of varying composition*

Lactose present (mg)	Glucose present (mg)	Lactose found (mg)	Glucose found (mg)	Lactose error (%)
1.8	0.6	1.6	0.9	-11
1.8	1.8	1.6	2.2	-11
1.8	6.0	1.6	6.0	-11
2.4	1.2	2.2	1.9	-8
2.4	2.4	2.3	2.7	-4
2.4	6.0	2.0	6.3	-17
3.0	12.0	2.9	11.2	-3
3.0	15.0	3.0	13.7	0
6.0	9.0	6.0	7.0	0
6.0	24.0	6.0	20.0	0
18.0	6.0	16.8	2.2	-7
18.0	12.0	17.6	8.5	-2

Estimation of lactose in solutions containing soluble nitrogen (tissue 'incubates') and salt mixtures

Colour development is partially inhibited in tissue 'incubates' unless such solutions are previously deproteinized. The use of strongly acid or alkaline reagents for deproteinization would clearly complicate the subsequent estimation, and in the search for a neutral precipitant zinc hydroxide (Letonoff, 1934) was tried and found most satisfactory. It has the additional advantage that its use involves no alteration in the volume of the solutions treated and no diminution of the lactose content. Approximately 0.1 g Zn(OH)₂ is added/30 ml of solution, and the mixture is allowed to stand for 5 min and then filtered. The filtrate is used for the estimation without further treatment. The addition of Zn(OH)₂ may be omitted when glycogen is present, since the ethanol also precipitates the protein.

Solutions pre-treated with Zn(OH)₂, or solutions containing Ca⁺⁺ or Mg⁺⁺ (e.g. Ringer phosphate solution), give a precipitate of insoluble hydroxides when NaOH is added during the estimation. In such cases the solutions are filtered through Whatman no. 1 filters at 9 min 30 sec (see time scheme for method).

DISCUSSION

We have extended Fearon's (1942) observations on the specificity of the lactose methylamine reaction and find that, in addition to maltose, cellobiose and certain lower dextrans give a similar colour. The dextrans are only slowly fermented by brewer's yeast and are thus readily distinguishable from maltose which is rapidly fermented. A limited investigation of their reduction values before and after acid hydrolysis, using the method of Somogyi (1945), gave ratios of approximately 1/5. It seems probable, therefore, that the specificity of the

reaction extends to disaccharides and lower poly saccharides containing the 1 4 glycosidic linkage, irrespective of their stereochemical relationships. In agreement with this, sucrose (1 2) and melibiose (1 6) fail to give the reaction, as do the trisaccharides raffinose and melezitose.

The method seems to offer scope for further application to mixtures of lactose with other sugars, either by direct measurement—when the contaminating sugar gives no colour (raffinose)—or by measurement at two selected wavelengths, when interfering colours are produced (galactose). Estimations in the presence of maltose or lower dextrans may be made after preliminary fermentation with brewer's yeast, though the slow and variable fermentation rates of the latter, presumably to be associated with differences in the degree of polymerization of the dextrans capable of giving a red colour, may present special difficulties. When

cellobiose is present an approximation to the correct lactose concentration can be made by first acting upon the solution with emulsin, which hydrolyses β glycosides much more rapidly than lactose.

SUMMARY

1 A method is described for the semi micro estimation of lactose, modifications are given for its application to solutions containing lactose in the presence of glucose and glycogen, and in tissue extracts, and more general recommendations made for its use when other contaminating sugars are present.

2 The method is most sensitive for lactose concentrations in the range 0.05–0.2 %.

We wish to express our thanks to Prof D C Harrison for his interest in the progress of this work and to Dr Q H. Gibson for valuable suggestions with regard to the graphical presentation.

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Displacement Chromatography on Synthetic Ion-exchange Resins

4 THE ISOLATION OF GLUCOSAMINE AND HISTIDINE FROM A PROTEIN HYDROLYSATE

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(Received 21 April 1949)

In Part 3 of this group of papers (Partridge, 1949) the fractionation of the hydrochloric acid hydrolysis product of commercial egg albumin was described. A displacement chromatogram, using a column packed with 'Zeo Karb 215' showed the presence of seven discrete bands each of which contained simple mixtures of amino acids. These were identified by carrying out partition chromatograms on filter paper by the method of Consden, Gordon & Martin (1944). Band VI contained histidine together with an unknown basic substance. This substance has now been identified as glucosamine and both glucosamine and histidine have been isolated from the amino acid mixture in a pure condition. The amount of glucosamine in the hydrolysis product is very small and its origin is probably the ovomucoid contained in white

of egg. Histidine is also present in small amount, the content of this amino acid in crystalline ovalbumin being about 2.4 % (Chibnall, 1945).

The following is the order in which the more basic amino acids appear in the effluent from a column of 'Zeo Karb 215' when ammonia solution is used as the displacement developer: leucine, histidine, glucosamine, lysine, ammonia. The affinity of glucosamine for the resin is not much greater than that of histidine, and separation is only partial with columns of normal length. It has been pointed out (Partridge & Westall, 1949; Davies, 1949) that when the displacement developer is a free base, the order of displacement reflects the ability of the stronger bases to control the pH of the aqueous phase and thus to depress the cationic form of the weaker bases.

Davies (1949) shows that the controlling equilibrium in the case of the amino acids may be represented by



In addition, there may be two further factors affecting the adsorption of an amino acid by the resin (a) van der Waals forces between the resin and the cation A^+ or the neutral molecule A^\pm , and (b) the affinity of the cation for the resin will be greater the higher its valency

It is clear, therefore, that by altering the environment of the ions in the ambient phase it would be possible to exaggerate the effect of one of these mechanisms at the expense of the others and thus to produce conditions in which the order of displacement is altered. Thus, by using a dilute solution of a salt as displacement developer the pH of the ambient fluid would be lowered and equation (1) would lie entirely to the left (in so far as univalent cations are concerned). This has been made use of to secure the separation of glucosamine from histidine. When a solution of a salt flows through a column containing an adsorbed mixture of amino acids, the cations of the salt are adsorbed and the anions are released together with the cations of the displaced amino acids. The high acidity thus set up results in the mechanism depending upon equation (1) becoming inoperative and the mutual displacement of the amino acids is thus controlled by mechanisms (a) and (b) only. In this case, as would be expected, the bivalent cations are strongly adsorbed and the observed order of displacement where 0.1 M sodium chloride is used as the displacement developer is as follows: glucosamine, Na^+ , leucine, histidine, lysine. The affinities of Na^+ and the leucine cation are very similar, but with long columns the Na^+ band tends to overtake that due to leucine. Lysine and histidine are much more strongly adsorbed than Na^+ , and the sodium band overruns these two amino acids and shows little tendency to cause their migration down the column by elution.

METHODS

Comparison of the effects of sodium chloride and sodium hydroxide solutions as displacement developers

The behaviour of the two displacement developers was compared directly by setting up two small identical columns and analysing with each the same sample mixture of bases.

The columns each contained 2 g of air dried 'Zeo Karb 215' (80–100 mesh/in., height of column 6.1 cm). Before use the columns were treated alternately several times with 2N-HCl and 2N NaOH. In both cases the mixture of solutes run through the column (in its hydrogen form) was histidine, 0.1 M, 10 ml, glucosamine hydrochloride, 0.1 M, 10 ml., glycine, 0.1 M, 10 ml.

Column (1) was developed by displacement with 0.1 N NaOH

applied at a rate of 1 ml/min. The effluent was collected in 3 ml fractions, 34 fractions being collected in all. A filter paper chromatogram was set up using a drop of solution from each fraction and the results are shown diagrammatically in Fig. 1a.

Column (2) was developed by passing at first a solution of NaCl (0.05 N) at a rate of 2 ml/min. A portion of the effluent (45 ml) was allowed to pass before commencing to collect fractions. After 34 fractions (each of 3 ml.) had been collected the reservoir containing NaCl solution was replaced by one containing 0.1 N-NaOH and the experiment was continued until a further 26 fractions had been taken. The composition of the effluent was analysed by paper chromatography as before and the results are shown in Fig. 1b.

The effluent solution at first contained HCl arising by removal of the cations from the NaCl solution, but when the column became saturated with cations the hydrogen ion concentration of the effluent fell rapidly and hydrochlorides of glycine and glucosamine appeared. Between fractions 10 and 14 (Fig. 1b) glucosamine and glycine hydrochloride were replaced by NaCl and the free acidity of the effluent fell to less than 0.001 N. On replacing the reservoir by one containing NaOH the residual NaCl solution was rapidly displaced and salt-free water flowed from the column until the appearance of the histidine band.

In order to collect data for the design of a suitable experimental procedure for the isolation of the components of the histidine-glucosamine band obtained in the primary separation, the experiment described above was repeated several times with small variations in the procedure. The results of these experiments may be summarized as follows.

(a) *Replacement of glycine by leucine* The experiment illustrated in Fig. 1b was repeated with leucine in place of glycine. The chromatogram showed a good separation between glucosamine and leucine, the leucine front almost coinciding with that due to sodium chloride. The leucine band had a very diffuse rear boundary, showing that, under the conditions of the experiment, the affinities of the leucine and sodium ions for the resin were similar. A small part of the leucine was not eluted from the column by the volume of sodium chloride solution applied, and this appeared as a sharp but narrow band in front of the histidine band when the histidine was displaced by sodium hydroxide solution.

(b) *Effect of increasing the concentration of sodium chloride* The sodium chloride concentration was increased to 0.1 M and the chromatogram compared with that shown in Fig. 1b. The result showed that while the glucosamine band was relatively shorter, and the glucosamine content of the effluent correspondingly higher, the leading edge of the band was very diffuse. This effect was probably due to the tendency of glucosamine to be eluted by the higher concentration of hydrochloric acid present.

(c) *Effect of replacing the sodium hydroxide solution by ammonia (0.1 N)* The resulting chromatogram showed an improvement in the sharpness of the histidine band. Since the use of sodium hydroxide

leads to difficulties where larger columns are employed, dilute ammonia solution was adopted for all further experiments

(d) *Effect of increasing the column length* The experiment was carried out using glucosamine hydrochloride, leucine and histidine as the experimental mixture. The increase in column length was about 25 % and the chromatogram showed an improvement in the separation of glucosamine and leucine. It was considered that the extra column length allowed the sodium band to overtake that due to leucine, with

grams carried out on the mixed solution showed this to contain histidine and the 'unidentified substance', together with smaller quantities of leucine, lysine and cystine

Identification of glucosamine

The unidentified substance was observed to have R_F values in a number of different solvents close to those given by glucosamine. Since the amino sugar gives a good colour reaction with ninhydrin and is known to occur in the ovomucoid of white of egg, the

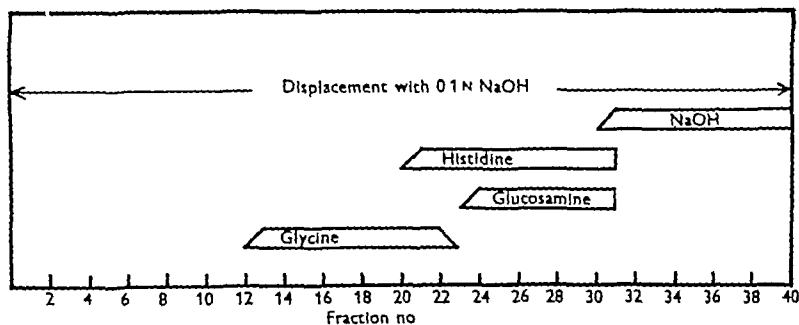


Fig 1a

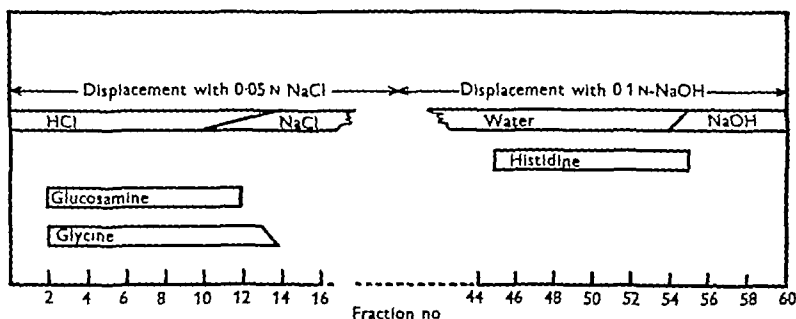


Fig 1b

Fig 1 a, Composition of the effluent from a column of 'Zeo Karb 215' on displacing a mixture of glycine, glucosamine, and histidine with 0.1N NaOH. b, Composition of the effluent obtained by displacing the amino acid mixture first with 0.05N NaCl followed by 0.1N NaOH

the result that a narrow zone of sodium chloride was interpolated between the glucosamine and the mixed sodium chloride leucine bands

Primary fractionation of the protein hydrolysate

A sample of commercial egg albumin (moisture 13.8 %, ash 3.4 %, 40 g) was hydrolysed with N hydrochloric acid. It was then treated with charcoal and fractionated on a column of 'Zeo Karb 215' exactly as described in Part 3 of this series (Partridge, 1949). Using the filter paper chromatogram as a guide, three fractions of the effluent (each of 47 ml) were combined together so as to comprise the whole of the histidine band. Further filter paper chromato-

identity of the two substances was probable. Like glucosamine, the unknown substance was shown to yield brown spots on chromatograms sprayed with ammoniacal silver nitrate and cherry red spots with the Elson & Morgan (1933) reagents (cf Partridge, 1948). Further chromatograms were carried out in phenol, collidine and butanol acetic acid mixture in which the unknown substance was compared directly with authentic glucosamine, and these confirmed the identity.

The only naturally occurring ninhydrin reacting substance known to have R_F values close to glucosamine in these three solvents is chondrosamine, but separation may be obtained between these two amino sugars on filter paper chromatograms by pro-

longed irrigation with collidine (cf Aminoff & Morgan, 1948). A few trials were required in order to determine satisfactory conditions for the separation of the two amino sugars, and the following procedure was finally adopted. A strip of Whatman no. 1 filter paper was cut to a point at one end and spots of glucosamine hydrochloride, chondrosamine hydrochloride, the unknown mixture and a mixture of the authentic amino sugars were applied to the starting line. When the spots were dry, a drop of concentrated ammonia solution was put on each of the spots and allowed to dry off. The chromatogram was then irrigated for 42 hr with collidine, allowing the solvent to drip off the pointed end of the paper. On drying and spraying with ammoniacal silver nitrate or the

A dilute solution of sodium chloride (0.05M, 200 ml) was applied at a rate of 2 ml/min and the effluent was collected in 20 ml fractions. A further quantity of more concentrated sodium chloride solution (0.1M, 400 ml) was then applied, after which the column was washed with water (5–10 ml) and the reservoir changed for one containing 0.15N ammonia. This solution (150 ml) was applied at a rate of 1 ml/min and the effluent collected in 10 ml fractions.

Fig. 2 shows the filter paper partition analysis of the effluent fractions. A good separation was obtained between glucosamine and leucine and between histidine and lysine, but the histidine fraction was contaminated by a little cystine. A series of very

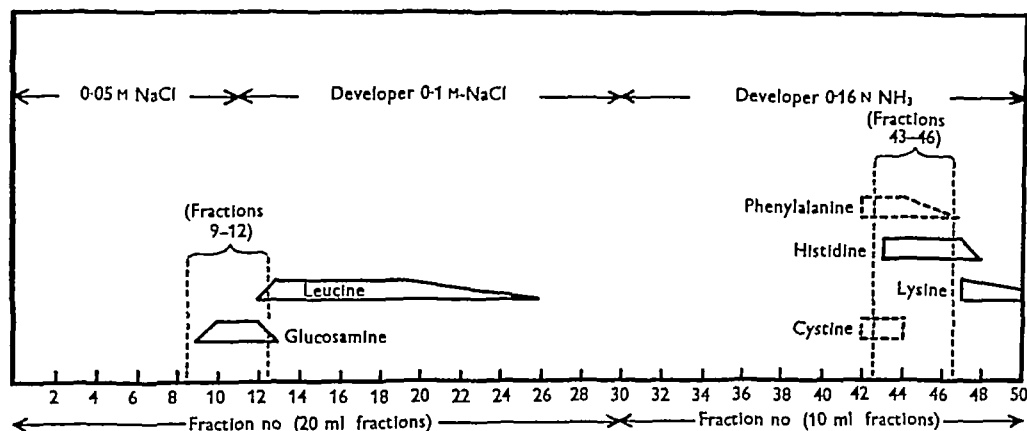


Fig. 2 Further fractionation of the histidine-glucosamine band obtained in the primary separation of the protein hydrolysate. The chromatogram shows the result of displacing the mixture from a column of 'Zeo Karb 215' with an increasing concentration of NaCl followed by ammonia solution.

Elson & Morgan (1933) reagents a clear separation was obtained between the two amino sugars, and chondrosamine was seen to be absent from the unknown mixture.

Isolation of glucosamine and histidine from band VI of the primary separation

The filtration tube was 13 mm in diameter and contained 10 g (dry wt) of 'Zeo Karb 215' (80–100 mesh/in). The column was prepared by alternate treatment with 0.15N ammonia and 2N hydrochloric acid in order to compact the bed (height occupied by the resin, 18.0 cm). The mixed solution obtained from band VI of the primary separation (140 ml) was applied to the column at a rate of 1.5 ml/min and the column was then washed with 10–20 ml water. Since the primary fractionation was carried out by displacement with 0.15N ammonia the concentration of bases in the mixed solution of band VI was about 0.09M (cf Part 1 of this series, Partridge, 1948), and thus the experimental mixture was sufficient to saturate rather less than half the new column (Part 1, fig. 3).

faint spots due to phenylalanine was noticed on the paper chromatogram. The bulk of this amino acid was removed by the preliminary charcoal treatment of the protein hydrolysate and it had not been observed in previous chromatograms carried out on the experimental solution, however, its appearance in the histidine band was probably due to the concentration of small traces by the chromatographic procedure. Fractions 9–12 (glucosamine hydrochloride) and 43–46 (histidine) were set aside for concentration and crystallization.

Crystallization of glucosamine hydrochloride. The mixed fractions 9–12 (80 ml) were evaporated to small bulk under reduced pressure and the syrup diluted with about 4 vol of methanol. The solution was then warmed, and warm acetone added until crystallization commenced. On cooling, a heavy crop of crystals separated (small prisms). Yield, 0.157 g, ash, 0.7%, N, 6.40% (calc for $C_6H_{14}O_5NCl$, N, 6.53%), $[\alpha]_D^{20} + 7.4$ (1 dm, c, 1.5 in water), yield as percentage of protein dry wt, 0.47. A chromatographic analysis of the product showed it to be free from amino acids.

Crystallization of histidine The mixed fractions 43-46 (40 ml) were concentrated to about 10 ml under reduced pressure and the solution stored at +2° overnight. It was then filtered to remove a light precipitate which was probably cystine. A small amount of charcoal was added and the solution warmed. It was then refiltered and the evaporation continued until a few crystals appeared in the liquid. An equal volume of warm ethanol was then added and a dense feltwork of crystals appeared on cooling. Yield, 0.303 g, ash, 0.3%, N, 26.5% (calc for $C_6H_9O_2N_3$, N, 27.1%), $[\alpha]_D^{20} - 32.6$ in water (c, 2.3, 1 dm), yield as percentage of protein dry wt, 0.92. Chromatographic analysis showed the product to be free from other amino acids.

DISCUSSION

The separation described above illustrates a point that has already been stressed in Part 3 of this series (Partridge, 1949), namely, that fractionation by displacement from an ion-exchange column has an advantage not possessed by the process of distillation from a fractionating column which it otherwise resembles in the chromatographic technique it is often possible to alter the adsorption affinity of a solute by altering the environment in which the adsorption takes place, thus a primary separation may be carried out in order to divide the components into a series of small groups, and each group may then be separated further by a secondary fractionation carried out with an alteration in experimental conditions calculated to produce a differential alteration in adsorption affinity.

Two distinct methods of using ion-exchange resins in displacement chromatography have been described here, displacement with a free base and displacement with a salt, the substitution of one method by the other leads to a remarkable change in the order in which the components leave the column. The differential technique has been used in this case to separate a univalent base, glucosamine, from a complex ampholyte, histidine, but, although no further experiments on these lines have yet been attempted, it is clear that a similar technique could be applied to other separations in which the com-

ponents are either different in charge structure or are well separated in a homologous series.

The isolation of glucosamine from a protein containing it in small amount has a special interest since many proteins are known to contain small quantities of hexosamines. Isolation by conventional procedures is a difficult matter, and although the hexosamine content of a protein may be estimated readily by colorimetric or other methods, none of these differentiates one hexosamine from another.

The yields of both glucosamine and histidine obtained in these experiments were low, probably not exceeding 40% in either case, but a large part of the loss occurred during the final process of crystallization which was carried out with 150-300 mg of material. Higher yields should be obtainable by increasing the scale.

SUMMARY

1 Glucosamine has been identified as a constituent of the hydrolysis product of commercial egg albumin.

2 A primary fractionation of the hydrolysate carried out by displacing the components of the mixture from a column of 'Zeo Karb 215' by means of dilute ammonia solution yielded a mixed band containing glucosamine and histidine together with other amino acids in small amount.

3 The components of the mixed band were again displaced from a smaller column of 'Zeo Karb 215' using dilute sodium chloride solution as the displacement developer. Glucosamine was displaced and was recovered in a pure condition as the hydrochloride. Histidine remained in the column.

4 The histidine was displaced from the column by means of ammonia solution and was recovered as the free base in a substantially pure condition.

5 The principle underlying the separation is discussed briefly and possible applications to other systems are pointed out.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The author wishes to acknowledge the assistance of Mr D F Elsdon.

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Enzyme Activities in the Blood of Infants and Adults

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(Received 13 May 1949)

Morphological studies of the blood of infants and adults have been fairly numerous, but functional or chemical comparisons have been rare. The circulating blood at birth has been shown to contain two kinds of haemoglobin, an infantile type which comprises four fifths of the whole and which is relatively resistant to alkaline denaturation, and the adult type which makes up the remaining fifth (Haurowitz, 1930, 1935, Brinkmann & Jonxis, 1935). Immunological differences between the adult and infant haemoglobins were detected by Darrow, Nowakovsky & Austin (1940). Whitby & Hynes (1935) found that concentrations of sodium chloride, which caused no haemolysis of adult red cells, readily haemolysed a proportion of the cells of a sample of infant blood. A concentration of sodium chloride, however, sufficiently dilute to cause total haemolysis of normal adult cells, did not disrupt all the cells in a sample of infants' blood. Such samples therefore contained some cells which were more and others less fragile than normal adult cells. Mollison (1948) compared the survival time in the infant circulation of red cells taken from cord blood with that of cells taken from an adult vein. He found that cord red cells disappeared at nearly twice the rate of the adult cells during the 10 days following transfusion. Stevenson (1943) estimated the carbonic anhydrase activity of infant and adult red cells and found it to be low in the former, and still lower in red cells obtained from premature infants, and Anselmino & Hoffmann (1931) claimed that the catalase activity of red cells derived from full-term infants was higher than that of maternal red cells. If all the morphological, physiological and chemical evidence is taken together it would appear that the red cells of an infant are a more heterogeneous population than those of an adult.

The present work was undertaken to compare the enzyme activities of the serum and erythrocytes of the newborn and adult human. The true and pseudo cholinesterase of the serum have been investigated, and in the erythrocytes true cholinesterase, glyoxalase, carbonic anhydrase, catalase and acid phosphatase.

METHODS

Preparation of serum and red cells. Blood was taken from the placental end of the divided umbilical cord or by puncturing the umbilical vessels with a large bore needle. Two samples were collected in sterile glass tubes or small bottles, one for the preparation of serum and the other, received into a few ml. of a solution containing 1 g. of sodium citrate dissolved in 100 ml. 0.9% (w/v) NaCl, for the separation of red cells. The specimens collected to provide serum were centrifuged as soon as the fibrin clot had separated. Haemolysed sera were not investigated. The citrated sample was centrifuged and the plasma removed. The cells were washed once with an equal vol. of 0.9% (w/v) NaCl, suspended in 0.9% NaCl and covered with a layer of liquid paraffin. In all manipulations care was taken to avoid bacterial contamination. Both the cells and the sera were stored in the dark at +4° until required. Keilin & Wang (1947) have shown that the activity of the enzymes under investigation was reduced by a very small amount when blood was similarly stored for many years, and no evidence of any loss of activity was found in the present work.

Cholinesterase was estimated in Warburg manometers at 37°. Two substrates were used (a) 0.135M acetylcholine chloride, and (b) 0.18M acetyl β methyl choline. The latter is said to be hydrolysed only by the so called true cholinesterase (Mendel, Mundle & Rudney, 1943). Since pseudo cholinesterase in human blood is thought to be confined to the serum, and the true enzyme almost entirely to the red cells, it seemed permissible to use the unspecific acetylcholine chloride as substrate for the serum enzyme.

The quantities of the reacting substances used to estimate cholinesterase activity are set out in Table 1. In each case

Table 1 *Quantities of reactants used in Warburg flasks to estimate blood cholinesterase activity*

Enzyme	Side bulb containing substrate	Main compartment	Unit of activity
Serum pseudo cholinesterase	0.135M Acetylcholine chloride, 0.5 ml	Serum, 0.1 ml, *Ringer bicarbonate, 0.9 ml	μ l. CO ₂ /min./ml. serum
Serum true cholinesterase	0.18M Acetyl β methyl choline, 0.5 ml.	Serum, 0.5 ml, 0.03M NaHCO ₃ , 0.5 ml	μ l. CO ₂ /min./ml. serum
Red cell true cholinesterase	0.18M Acetyl β methyl choline, 0.5 ml	Lysed red cells, 0.2 ml, *Ringer bicarbonate, 2.3 ml	μ l. CO ₂ /min./mg. Fe

* Bicarbonate solution of Krebs & Henseleit (1932)

the manometers were gassed with a mixture of 5% CO₂ and 95% of O₂ (v/v), and equilibrated until two successive readings were constant. With the quantities of reactants given, CO₂ evolution was a linear function of time over the period of the experiment. Readings were taken at 2.5 min intervals for 15 min except when the true cholinesterase was being estimated in the serum. Readings were then taken at 10 min intervals for a period of 1 hr.

Column 4, Table 1, defines the unit of activity employed. In the red cells iron was estimated by the method of Lyons (1927), described by McCance, Widdowson & Shackleton (1936), and the activity of the enzymes was calculated/mg of Fe.

Glyoxalase activities were measured in Warburg manometers at 37°. 0.5 ml of a 0.0139*N* solution of methyl glyoxal, which had been neutralized in bulk with a few drops of Na₂CO₃, was used as substrate. The main chamber of the Warburg flask contained 0.1 ml of diluted lysed red cell solution, 0.65 ml of Ringer bicarbonate (Krebs & Henseleit, 1932) and 0.25 ml of 0.36% glutathione. The gas space was filled with a mixture of 5% CO₂ and 95% O₂ (v/v). After equilibration six readings were taken at 2.5 min intervals over which period CO₂ evolution was linear. The results were based on the iron content of the sample and the activity was expressed in μ l CO₂ evolved/min/mg Fe.

Carbonic anhydrase activity was measured by the 'boat' method of Meldrum & Roughton (1933). Experiments were conducted at 16.0 \pm 0.2°. The activity was calculated from the formula given by the authors, and the result was expressed in their arbitrary units/mg Fe in the sample of red cells under investigation.

Catalase. George (1947) showed that in the reaction between catalase and H₂O₂, O₂ evolution takes place in two phases. An initial rapid phase, of about 30 sec duration, is followed by a slower phase. He claimed that the rate of O₂ formation in the rapid phase was proportional to the enzyme concentration. These observations formed the basis of the method employed to compare the activity of catalase in adult and infant red cells. The Meldrum & Roughton (1933) 'boat' apparatus was used. 1.5 ml. of 0.2*M* phosphate buffer, pH 6.5, and an equal vol. of diluted lysed red cell solution were added to one side of the boat, and 1.5 ml of 0.4*M* H₂O₂ to the other. After a period for equilibration with temperature and pressure, the 'boat' was shaken violently

in the apparatus and the O₂ evolution followed to completion manometrically. Experiments were performed at 16.0 \pm 0.2°. Experiments in which water was substituted for the red cell solution showed an insignificant O₂ evolution. The blank value was therefore neglected. O₂ evolution over the initial rapid phase of the reaction was linear for about 30 sec. All the results were calculated from the pressure difference after 20 sec. The results are expressed/mg of Fe in the solution of red cells under investigation.

Acid phosphatase was estimated in red cells by the technique of King (1947). The unit of activity used is a modification of that defined by King, Wood & Delory (1945) and represents mg of phenol liberated under the conditions of the experiment in 1 hr/mg Fe in the sample of red cells under investigation.

Haemoglobin was estimated by the method of Gibson & Harrison (1946).

RESULTS

A summary of the results obtained for the seven enzymes examined is set out in Table 2. The mean activity of each enzyme, with the exception of acid phosphatase, was lower in infancy than in adult life. The differences were statistically significant. In the case of the red cell acid phosphatase there was no significant difference between the mean values of the adult and infant group.

The values recorded for carbonic anhydrase confirmed the finding of Stevenson (1943), who showed the activity of infant blood to be lower than that of the adult, and although the unit used by him was not identical with that of the present work the difference was in the same ratio (roughly 1/3). We also confirmed his observation that the activity of the red cells in some newborn infants is almost immeasurably low. He also stated that the carbonic anhydrase activity of the erythrocytes of premature babies (1500 g or less) was low when compared with the corresponding values in full term infants. In the present series the birth weights varied between 2150 and 4470 g, but there was no evidence that a low

Table 2 Comparison of enzyme activities in serum and red cells of adult and cord blood

Enzyme and unit of activity	Age and no of samples	Mean activity and s.e.	<i>t</i>	Degree of significance (<i>P</i>)
Serum pseudocholinesterase	Adult 24	76.7 \pm 2.82	6.43	<0.001
Unit = μ l CO ₂ /ml/min	Infant 24	50.2 \pm 2.94		
Serum true cholinesterase	Adult 24	1.053 \pm 0.055	3.22	<0.005
Unit = μ l CO ₂ /ml/min	Infant 24	0.827 \pm 0.043		
Red cell true cholinesterase	Adult 11	48.31 \pm 5.49	3.3	<0.005
Unit = μ l CO ₂ /mg Fe/min	Infant 24	32.90 \pm 1.94		
Red cell carbonic anhydrase	Adult 13	36.65 \pm 2.51	14.8	<0.001
Arbitrary units/mg Fe \times 10*	Infant 26	9.10 \pm 2.41		
Red cell catalase Arbitrary	Adult 13	27.67 \pm 2.39	2.89	<0.01
units/mg Fe	Infant 27	19.23 \pm 1.58		
Red cell glyoxalase μ l CO ₂ /mg Fe/min	Adult 7	555.0 \pm 75.2	3.57	<0.005
	Infant 9	281.0 \pm 34.2		
Red cell acid phosphatase	Adult 11	4.61 \pm 0.423	0.375	Difference not significant
Arbitrary units/mg Fe	Infant 9	4.84 \pm 0.617		

The difference is considered to be statistically significant when *P* < 0.05

birth weight was associated with low, or for that matter with high, carbonic anhydrase activity

Estimation of catalase showed that infant red cells had a lower mean activity than adult red cells, and did not confirm the work of Anselmino & Hoffmann (1931), who compared the catalase activity of cord blood with that of maternal blood. However, these authors used a method which involved the estimation of residual hydrogen peroxide by permanganate titration, which cannot be assumed to give results comparable with those given by the present technique. There is, for example, no evidence that the activity measured by permanganate titration is equivalent to that measured over the initial part of George's (1947) biphasic reaction, or that the catalase activity of maternal blood at term is the same as that of the non-pregnant adult.

DISCUSSION

The average haemoglobin found in twenty two samples of cord blood was 15.6 g/100 ml and the average value for the adults was 15.9 g/100 ml. The cord blood, however, contained 54.3% of cells by volume and the adult blood 49.5%. The enzyme activities of the cells are expressed/mg of iron, but since the percentage of iron in infant and adult haemoglobins is thought to be the same (Best & Taylor, 1945; Haurowitz, 1930, 1935) the activities of the enzymes can be expressed/g of haemoglobin without altering their relationships. If, however, the enzyme activities had been expressed/unit volume of red cells the differences found would have been magnified because the infant cells contained less haemoglobin/unit volume. Thus the mean activity of the erythrocyte cholinesterase in adults was 52.25 units/ml of cells, as against 48.31 units/mg of iron, and in infants 31.8 units/ml of cells against 32.9 units/mg of iron.

It is difficult to decide what significance should be attached to the low enzyme activities of the red cell at birth. It is important to recognize that the erythrocyte, even at this time of life, is a highly specialized cell. Any chemical activity which it manifests, therefore, must be either a legacy of the haemopoietic mechanism which formed it, or an expression of the metabolism of a mature cell. If the erythrocyte enzymes investigated are involved in the haemopoietic processes, variations in their activity may be the cause of some of the differences in chemical composition which are known to exist between the adult and the infant red cell. Differences in activity might also affect the rate of haemopoiesis, and Rittenberg & Shemin's (1946) work suggests

that the lower activity of the infant enzymes need not necessarily imply a slower rate of haemopoiesis. But until the differences in chemical composition and the factors controlling haemopoiesis in the adult and the infant are better understood such arguments must remain largely speculative. If, on the other hand, the erythrocyte enzymes are involved in the maintenance of the cell while it is in the circulation, it should ultimately be possible to correlate the differences of activity which have been demonstrated in adult and infant erythrocytes with the survival of these cells in the circulation. On this hypothesis, indeed, lower activities in the infant erythrocyte might have been inferred from Mollison's (1948) observations that transfused cord red cells disappeared more rapidly from the circulation than did adult cells. The integrity of the red cell must depend on the preservation of its membrane, and in this connexion the observation of Paleus (1947) is interesting, for he claimed that the erythrocyte cholinesterase was localized in the membrane.

The foregoing discussion should not be considered to embrace carbonic anhydrase, since its function in carbon dioxide transport and in the 'chloride shift' is well established, although the significance of its very low activity in the infant red cell is still obscure.

So little is known of the physiological function of the serum cholinesterases, that attempts to interpret the present findings seem neither profitable nor justifiable.

SUMMARY

1 The mean cholinesterase activity of sera obtained from cord blood was 50.2 units for the 'pseudo' enzyme and 0.827 unit for the 'true' enzyme. The mean values for normal adult sera were 76.7 units and 1.053 units respectively. The differences were statistically significant.

2 The activities of the erythrocyte enzymes, true cholinesterase, glyoxalase, carbonic anhydrase, and catalase obtained from cord blood were found to be 32.9, 281, 9.1×10^2 and 19.23 units/mg iron. The corresponding values for normal adults were 48.3, 555.36×10^2 and 27.7 units/mg iron. All differences were statistically significant.

3 The erythrocyte acid phosphatase activity was measured in cord blood and was found to be 4.84 units/mg iron, the adult control group showed a value of 4.61 units. The difference was not statistically significant.

Estimations of enzymic activity in some of the controls were done by Miss A. O. Hutchinson to whom our grateful thanks are due.

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The Fate of Certain Organic Acids and Amides in the Rabbit

9 LOWER ALIPHATIC AMIDES

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(Received 9 May 1949)

In an earlier investigation, reported in Part 3 of this series (Bray, James, Ryman & Thorpe, 1948), it was shown that rabbit liver extracts were only slightly active in hydrolysing formamide, acetamide, propionamide and phenylacetamide. It had previously been shown (Bray, Neale & Thorpe, 1946) that phenylacetamide was apparently completely hydrolysed in the intact rabbit, since its excretion products were qualitatively and quantitatively virtually the same as those of phenylacetic acid. In the case of the aromatic amides for which information is available (Bray, Thorpe & Wood, 1949a) there is reasonable agreement between results *in vivo* and *in vitro*, so that it seemed desirable to investigate the metabolism of aliphatic amides in greater detail.

These compounds have, in general, attracted little attention, probably because of the difficulty in determining their fate. Schultzen & Nencki (1872) concluded that acetamide was not hydrolysed in the dog. This was supported by Steinhausen's (1914-20) finding that in the phlorrhizinated dog 72% of a dose of the amide was excreted unchanged over a period of 6 days. In the rabbit, however, acetamide appeared to be largely hydrolysed, since Salkowski (1877-8) could detect only a small proportion of unchanged amide in the urine, and Rudzski (1876) claimed that a large proportion of a dose was

excreted as acetic acid. Pommerrenig (1902) reported that small doses were completely catabolized. Indirect evidence that acetamide is slowly and only partially hydrolysed in the rat has been obtained recently by Anker & Raper (1948) in feeding experiments with acetamide labelled with ^{13}C . Halsey (1898) found that formamide gave rise to as much urinary formate in the dog as did formic acid itself, and was therefore presumably completely hydrolysed, while Gonnermann (1902) found that both formamide and acetamide were hydrolysed by sheep liver and formamide by sheep kidney. The other amides studied here do not appear to have been investigated previously, except by Fiske (1923), who carried out a study of their hydrolysis by fasting cats, using urinary amide nitrogen estimation as the criterion of splitting. Acetamide was excreted over about 4 days, a total of 70% of the dose appearing unmodified. With the straight chain amides up to valeramide, the lag in excretion diminished and the extent of hydrolysis increased progressively with increasing molecular weight, *n* valeramide being almost completely hydrolysed (95-96%) within 24 hr. The extent of hydrolysis of propionamide was 55-57% and of *n* butyramide 74-80%. The order of stability *in vivo* was the reverse of that towards alkali, with which reagent the degree of hydrolysis

decreases as the molecular weight increases. In our earlier study (Bray *et al.* 1948) we found that shorter chain aliphatic amides in general were much more readily hydrolysed by alkali than by liver extracts.

This paper reports the results of an investigation upon the amides of straight chain saturated fatty acids from formic to valeric, in which the excretion of unchanged amides was estimated in terms of the increased ether soluble acid formed as a result of hydrolysis, the hydrolytic effect of rabbit liver extracts and slices was also observed.

EXPERIMENTAL

A *IN VIVO* EXPERIMENTS

Methods and materials

Diet and dosage. The rabbits and diet were as previously described (Bray, Ryman & Thorpe, 1947). The amides were administered by stomach tube as solutions or suspensions in water and the acids as solutions of their sodium salts. At the dose levels used none of the compounds showed toxic properties.

Estimation of amides. Owing to the lack of suitable characteristic properties, the amides could only be estimated by a non specific method. A procedure based on the liberation of NH_3 by alkaline hydrolysis was unsatisfactory and the method finally adopted involved estimation of the increase in the ether soluble acid after hydrolysis.

Urine (50 ml) was hydrolysed by addition of 10N NaOH (5 ml) and heating for 2 hr in a boiling water bath, the pH was adjusted to 8, using thymol blue as indicator, the urine cooled and the volume adjusted to 100 ml. The ether soluble acid content was then estimated as below. By this method 95–102% of added amides were recovered from normal rabbit urine.

Estimation of ether soluble acid. Ether soluble acid was extracted as previously described (Bray *et al.* 1947). Since, however, all the aliphatic acids in this study are volatile, the ether in the 'boiler' was not evaporated as usual but titrated directly after the addition of water (20 ml), thymol blue being the indicator. 'Fixing' the extracted acid by addition of excess standard NaOH before evaporation was undesirable in estimations on unhydrolysed urines, since it has been shown that an error may be introduced owing to hydrolysis of unchanged amide during evaporation (e.g. phenylacetamide, cf. Bray *et al.* 1946).

Table 1 *Excretion of aliphatic acids and amides by the rabbit**

Compound	Dose (g)	No of exps	Percentage of dose estimated as ether soluble acid in urine (Ranges in parentheses)		Estimated percentage hydrolysis in rabbit†
			Unhydrolysed	Hydrolysed	
Sodium formate	2–3	5	4 (0–7)	7 (4–12)	—
Formamide	2	3	5 (0–6)	43 (41–45)	60 (55–62)
	3	3	5 (2–8)	48 (47–50)	56 (54–58)
	4	2	2 (1–2)	33 (29–36)	69 (66–72)
		8	Av 3	43	61
Sodium acetate	2–5	7	1 (0–4)	1 (0–7)	—
Acetamide	1.5	1	0	71	29
	2	4	1 (0–4)	65 (44–78)	37 (22–56)
	2.5	3	2 (0–5)	75 (58–87)	27 (13–47)
	3	4	0	80 (62–94)	20 (6–38)
	5	4	1 (0–3)	72 (62–88)	29 (12–41)
		16	Av 1	73	28
Sodium propionate	2–3	4	0	0	—
Propionamide	2	4	3 (0–6)	47 (36–59)	56 (50–64)
	3	2	0	52 (48–55)	49 (45–52)
	4	2	0	48 (48)	52 (52)
		8	Av 1	48	53
Sodium <i>n</i> -butyrate	2–3	4	0	0	—
<i>n</i> Butyramide	2	3	0	8 (0–16)	92 (84–100)
	3	4	0	17 (12–24)	83 (76–88)
		7	Av 0	13	87
Sodium <i>n</i> valerate	2	4	0	0	—
<i>n</i> Valeramide	1.5	2	0	6 (0–12)	94 (88–100)
	2	2	0	0	100
	3	2	2 (0–3)	5 (0–10)	97 (93–100)
		6	Av 1	4	97

* The values given for the excretion of amides represent the total excretion, in some cases over periods up to 72 hr after dosage (cf. Table 2).

† Calculated from individual values.

RESULTS

Ether soluble acid of unhydrolysed and hydrolysed normal rabbit urine The average ether soluble acid excretion by individual rabbits ranged from 661 to 850 mg/24 hr, expressed as hippuric acid. The ether soluble acid content of hydrolysed urine is conveniently expressed as a percentage of the value for unhydrolysed, it ranged from 148 to 200%. The average for 107 experiments was 171%, the average for individual rabbits ranged from 157 to 181%. The day to day variations in output for each rabbit was usually within $\pm 10\%$ of the mean values for both unhydrolysed and hydrolysed urines.

Excretion of aliphatic acids and amides by the rabbit Table 1 summarizes the results obtained, taking the difference between the amount of amide administered and the total amount excreted unchanged as a measure of the extent of hydrolysis. The results with acetamide, propionamide, butyramide and *n*-valeramide are in general agreement with those of Fiske (1923), the degree of hydrolysis increasing with increasing molecular weight. Formamide is an exception, apparently being hydrolysed to a greater extent than acetamide. The possibility of some other type of catabolic process for formamide has not, however, been excluded. Proof of the presence of two of the amides unchanged in the corresponding urine has been obtained by isolation from chloroform extract (acetamide) or the ether extract (propionamide).

No evidence of the excretion of the acids was observed after the first 24 hr, but there was a delayed excretion of some of the amides beyond 48 hr after administration of the dose. Table 2 shows the percentages of the doses of the amides excreted unchanged in 24 hr periods after the dose. Here also we are in general agreement with Fiske (1923). The lag period decreases with increasing molecular weight, and hence with increasing degree of hydrolysis.

Table 2 Rate of excretion of aliphatic amides by the rabbit

Amide	Dose (g)	Average percentage of amide excreted unchanged in 24 hr periods after dose			
		1st	2nd	3rd	Total
Formamide	2.0-4.0	27	8	4	39
Acetamide	1.5-5.0	62	6	4	72
Propionamide	2.0-4.0	37	10	0	47
<i>n</i> -Butyramide	2.0-3.0	13	0	0	13
<i>n</i> -Valeramide	1.5-3.0	3	0	0	3

B IN VITRO EXPERIMENTS

Methods

Buffer solution All the experiments were carried out at 37° at pH 7.4 in a phosphate buffer consisting of 0.2M Na_2HPO_4 (8 ml) and 0.2M NaH_2PO_4 (2 ml).

Preparation of digests (a) Slices were cut from the liver immediately after removal from the animal and placed in the buffer. The required amount (8 g) was made up to 10 ml with buffer and added to the substrate in oxygenated buffer to make a tissue concentration equivalent to that of the extracts. The digests were incubated in large flasks filled with O_2 .

(b) Extracts were prepared as previously described (Bray *et al* 1948). Extract (10 ml) was added to the substrate (0.01204M) in buffer (50 ml). In experiments lasting more than 6 hr it has been our practice to add CHCl_3 as a preservative. It will be seen in Table 3 that this retards the rate of hydrolysis, which was greater in 5 hr without than in 20 hr with CHCl_3 . In general, the constancy of controls could not be relied upon for more than 6 hr in the absence of preservative.

Estimation of amide hydrolysis The NH_3 formed was estimated by the method previously used (Bray, James, Raffan, Ryman & Thorpe, 1949b). In each experiment a control consisting of tissue or extract and buffer without substrate was included. A digest containing *p*-nitrobenzamide was also always included as a check upon the amidase activity of the liver.

Stability of substrates All the amides examined were stable in the buffer and no formation of NH_3 was observed when they were incubated with boiled liver extracts.

Table 3 Hydrolysis of aliphatic amides by rabbit liver extracts

Percentage hydrolysis (Ranges in parentheses. Superior figures against ranges indicate the number of experiments)

	With CHCl_3			Without CHCl_3	
	3 hr	5 hr	20 hr	3 hr	5 hr
Formamide	4 (3-5) ⁷	4 (3-5) ⁴	4 (0-9) ⁶	1 (0-3) ⁴	4 (2-5) ⁴
Acetamide	3 (0-4) ³	—	3 (0-7) ³	—	2 (1-3) ²
Propionamide	1 (0-4) ²	2 (0-3) ²	5 (3-8) ⁴	1 (0-2) ²	3 (0-5) ²
<i>n</i> -Butyramide	4 (1-6) ⁴	6 (6) ¹	9 (3-14) ⁴	8 (4-12) ³	13 (6-18) ⁷
<i>n</i> -Valeramide	11 (4-19)	13 (4-27) ³	39 (29-45) ⁴	48 (34-83) ⁵	55 (41-85) ⁵
Phenylacetamide	2 (0-5) ³	6 (3-8) ²	7 (0-19) ³	15 (8-20) ⁴	22 (12-32) ⁴
<i>p</i> -Nitrobenzamide	39* (17-49) ⁷	46* (17-58) ⁷	61* (29-81) ⁷	71 (56-92) ¹¹	79 (67-92) ¹¹

* These values should not be compared with those given in a previous paper (Bray *et al* 1948) which were obtained with 0.00637M *p*-nitrobenzamide.

Hydrolysis of aliphatic amides by rabbit liver extracts The degree of hydrolysis is given in Table 3, which also shows the retardation due to use of chloroform as preservative

Hydrolysis of aliphatic amides by rabbit liver slices The degree of hydrolysis is shown in Table 4

Table 4 *Hydrolysis of amides by rabbit liver slices*

Amide	No of exps	Percentage hydrolysis (ranges in parentheses)		
		2 hr	5 hr	6 hr
Formamide	3	8 (4-13)	10 (6-17)	10 (6-17)
Acetamide	5	0 (0-2)	1 (0-3)	1 (0-3)
Propionamide	4	3 (0-6)	5 (3-7)	5 (3-7)
n Butyramide	5	18 (15-22)	35 (31-39)	38 (35-43)
n Valeramide	4	37 (30-43)	65 (49-75)	70 (52-81)
Phenylacetamide	5	12 (9-19)	28 (19-36)	29 (22-40)*
p Nitrobenzamide	7	37 (30-50)	66 (53-76)	70 (56-82)

* Four experiments only

Comparison of the hydrolysis of amides by rabbit-liver extract and slices The percentages of p nitrobenzamide, n butyramide, n valeramide and phenylacetamide hydrolysed by extract and by slices prepared from the same liver are given in Table 5. It will be seen that there are appreciable differences between the degrees of hydrolysis brought about by extracts and by slices. It is difficult to assess the significance of these differences for p nitrobenzamide and n valeramide, but for n butyramide and

phenylacetamide they are considerable. It may also be noted that with p nitrobenzamide there are considerable differences in the initial rate of hydrolysis.

DISCUSSION

Table 6 summarizes the results of both *in vivo* and *in vitro* studies. The results of the former for acetamide, propionamide, n butyramide and n valeramide are in good agreement with those of Fiske (1923) for fasting cats, the degree of hydrolysis

Table 6 *Hydrolysis of amides in the rabbit and by rabbit liver slices and extract*

Amide	Percentage hydrolysis by		
	Intact animal	Extract (5 hr)	Slices (5 hr)
Formamide	61*	4	10
Acetamide	28*	2	1
Propionamide	53*	3	5
n Butyramide	87*	13	35
n Valeramide	97*	55	65
Phenylacetamide	100†	22	28
p Nitrobenzamide	100‡	79	66

* Based on recovery of unchanged amide

† Only 71% of dose accounted for, but metabolism almost identical with that of phenylacetic acid (Bray *et al.* 1946)

‡ Bray *et al.* (1949a)

increasing with chain length and the lag in excretion decreasing. Formamide is an exception, being hydrolysed to a much greater extent than acetamide, although the rate of excretion is comparable. The lag in excretion (Table 2) is probably not related to delayed absorption of the amides, since Höber & Höber (1937) found that in rat intestine there was little difference between the absorption rates of

Table 5 *Comparison of hydrolysis of amides by rabbit liver extracts and slices*

		Percentage of hydrolysis of							
Exp	Time (hr)	p Nitrobenzamide		n Butyramide		n-Valeramide		Phenylacetamide	
		Extracts	Slices	Extracts	Slices	Extracts	Slices	Extracts	Slices
1	0.5	19	13	—	—	15	10	1	2
	2	48	39	—	—	36	33	6	9
	4	65	62	—	—	48	56	10	16
	6	71	71	—	—	54	66	12	22
2	0.5	23	7	—	—	7	7	—	—
	2	47	30	—	—	22	30	—	—
	4	63	47	—	—	38	44	—	—
	6	73	56	—	—	43	52	—	—
3	0.5	32	17	2	8	—	—	3	6
	2	62	50	7	22	—	—	11	19
	4	72	70	12	32	—	—	17	31
	6	88	82	16	38	—	—	20	40
4	0.5	37	—	2	6	—	—	—	—
	2	64	—	9	20	—	—	—	—
	4	77	—	15	34	—	—	—	—
	6	81	—	19	43	—	—	—	—

acetamide and *n*-valeramide. The explanation more probably lies in the rate of reabsorption of these compounds in the kidney. The earlier finding (Bray *et al.* 1948) that in the case of phenylacetamide there is less correlation between the degree of hydrolysis *in vivo* and by extracts *in vitro* than with aromatic amides is shown by the present investigation to apply to other aliphatic amides. The amides studied were, with the exception of *n*-valeramide, hydrolysed to a much greater extent *in vivo* than would be expected from their *in vitro* hydrolysis by extracts. It is, however, possible to offer an explanation for this discrepancy, taking into account the observed lag in excretion of formamide, acetamide and propionamide. In these three amides it may be suggested that the extra reaction time allowed by the delay in excretion compensates for the feeble hydrolytic power of the enzyme responsible. This factor does not operate with *n*-butyramide, but there is instead evidence of more efficient enzyme action provided by the experiments with slices. In these experiments (Tables 4 and 5) the conditions used presumably approximate somewhat more closely to those *in vivo*, and the results show that the hydrolytic activity is much greater than in extracts. With phenylacetamide the percentage hydrolysis observed *in vitro* is 22–29% compared with an *in vivo* hydrolysis of 100% (Bray *et al.* 1948). In the earlier investigation, however, it was shown that there was a delayed excretion of the metabolites of phenylacetamide in the rabbit. Thus in the case of all five amides there are secondary factors which may be interpreted as accounting to some degree for the discrepancy between the observed *in vivo* and *in vitro* results. It should, however, be remembered that the *in vivo* results for formamide, acetamide, propionamide, *n*-butyramide and *n*-valeramide were obtained by determination of the amount of amide excreted unchanged. In the case of *n*-butyramide and *n*-valeramide the *in vitro* experiments indicate that

hydrolysis of these amides occurs, but there is no significant enzymic hydrolysis of the other three. This leaves open the possibility that these three amides may be metabolized by some process other than simple hydrolysis, or in some organ other than the liver. Whether the enzyme responsible for the hydrolysis of aliphatic amides is the same as that which hydrolyses aromatic amides is not yet certain. The evidence at present available indicates that the hydrolysis of the aliphatic amides is depressed to a somewhat greater extent by chloroform than is that of *p*-nitrobenzamide. The possibility that different enzyme systems may be involved is being explored.

SUMMARY

1 The metabolism of formamide, acetamide, propionamide, *n*-butyramide, *n*-valeramide and their parent acids has been studied in rabbits by estimating the extra ether soluble acid in unhydrolysed and hydrolysed urines after administration of the compounds.

2 The percentages of the doses of the amides excreted unchanged were 39, 72, 47, 13 and 3, respectively.

3 There is a considerable lag in the excretion of formamide and acetamide and a smaller lag with propionamide.

4 The hydrolysis of the above amides and phenylacetamide by rabbit liver extracts and slices has been compared. Formamide, acetamide and propionamide were only hydrolysed to a very small extent. The degrees of hydrolysis for *n*-butyramide, *n*-valeramide and phenylacetamide were for extracts, 13, 55 and 22%, and for slices, 35, 65 and 28%, respectively.

The authors are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this investigation.

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The Biochemistry of Locusts

2 CAROTENOID DISTRIBUTION IN SOLITARY AND GREGARIOUS PHASES OF THE AFRICAN MIGRATORY LOCUST (*LOCUSTA MIGRATORIA MIGRATORIOIDES* R & F) AND THE DESERT LOCUST (*SCHISTOCERCA GREGARIA* FORSK)

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(Received 13 May 1949)

The tremendous destruction which can be caused by locust plagues is widely appreciated, but, although locust research has attracted the interest of a large number of workers, the biochemical aspects of the problem have received little attention.

Uvarov (1921) was the first to appreciate the significance of the different forms of *Locusta migratoria*, and his theory of phases as applied to Acrididae which swarm is now universally accepted. In the *solitaria* phase locusts live in isolation, this implies that they are independent of their neighbours rather than that they are particularly scarce. In the *gregaria* phase hoppers (nymphs) occur in dense bands, keep close to each other, and march together. Gregarious adults form vast swarms which keep together even in flight. The two extreme phases are connected by a complex series of transitional forms which are descriptively named *transiens*. Factors which are known to influence the transition from solitary to gregarious forms and vice versa have recently been discussed by Uvarov (1947). The main factor controlling phase transformation is probably the population density, when solitary locusts are brought together they gradually change into the gregarious phase which is most strikingly evidenced by the insects becoming quite different in colour. The effect of crowding is in all probability to increase the activity of hoppers owing to frequent encounters with their neighbours. This increased activity appears to be the fundamental cause of the colour changes, for a solitary hopper maintained in isolation will also assume gregarious characteristics when stimulated to continuous exertion (Husam & Mathur, 1936).

After hatching, *L. migratoria* and *Schistocerca gregaria* (the two species under present consideration) normally go through five hopper stages, moulting at the end of each, and after about 22 days (at 32°) finally emerge as winged adults, under optimal conditions, from 2 to 4 weeks after emergence the adults become sexually mature (Uvarov, 1928).

Gregarious hoppers of *L. migratoria* are characterized in the early stages by their dark appearance which is broken by yellow or brown spots and stripes, in the later stages orange brown predominates, although there are still considerable

regions of black pigmentation. At adult emergence there appears to be little black pigment and the colour is greyish brown, but on reaching maturity the males become bright yellow and the females dark brown. The overall colour pattern is very stable. The coloration of solitary *Locusta*, on the other hand, varies considerably, solitaires are generally lighter coloured than *gregaria* and tend to adopt the colour of their environment (Faure, 1932). They contain little, if any, black pigment and do not undergo colour changes at maturity.

Solitary and gregarious *Schistocerca* exhibit very much the same characteristics as do the corresponding *Locusta*, although the pigment pattern is different. The coloration of gregarious hoppers is very stable and in the later stages the insects are lemon yellow with very pronounced black patterns. Immature adults are generally pinkish with small dark spots, especially on the elytra, and they turn to yellow when maturity is reached. The colour of solitary *Schistocerca* is much more variable than that of gregarious *Schistocerca*, in general, hoppers are green, immature adults greenish white, and mature adults grey (Uvarov, 1928). Some appreciation of these colour variations in *Schistocerca* and *Locusta* can be obtained by examining the coloured plates produced by Faure (1932).

It will be obvious from the above very brief survey that investigations into the biochemistry of locust pigmentation are essential to a deeper understanding of the phase transformation. The pigments which are stated to occur in locusts are melanin (Chauvin, 1937, 1939*a*), acridoxanthin, a pigment of unknown constitution (Chauvin, 1938*a, b*, 1939*b*, 1940, 1941*a, b*, 1944), carotenoids (Lederer, 1935, Chauvin, 1941*b*, Goodwin & Srisukh, 1948, 1949), an unidentified green pigment variously described as a chlorophyll derivative (Chauvin, 1939*a*) and as a flavone (Chauvin, 1941*b*), flavins (Gourevitch, 1937, Drilhon & Busnel, 1939, Busnel & Drilhon, 1942, Drilhon, 1943), and, possibly, a pterin (Busnel & Drilhon, 1942).

In the present investigation attention has been confined to the two carotenoids, β carotene and astaxanthin, which occur in both species (Goodwin & Srisukh, 1948, 1949), and the results of a study of

their quantitative distribution in *Locusta* and *Schistocerca* at various stages of development and in both the solitary and gregarious phases are now reported. A similar investigation on the distribution of acridoxanthin will be reported later

MATERIAL AND METHODS

Two species of locusts have been studied the African migratory locust (*Locusta migratoria migratoroides* R & F) and the desert locust (*Schistocerca gregaria* Forsk). The locusts were bred in captivity at the Anti Locust Research Centre in London, they were maintained under reasonably constant conditions and fed entirely on grass and wheat bran. The only difference between the breeding of the solitary and the gregarious phases was that the solitaires were completely isolated (one in a 3 lb jam pot of capacity about 1 l), whilst the gregarious insects were reared in crowds (about 50/jam pot or at high densities in cages up to 150 l. in capacity). Specimens were sent to Liverpool by post and generally arrived in good condition, any dead insects were discarded. In order to determine pigment distribution the insects were dissected into three portions head (comprising the head and pronotum), body and legs, the wings, which only contain relatively small amounts of carotenoids (Goodwin & Srisukh, 1949) were not examined systematically. The insects were killed with ether, although occasionally this step was omitted, and the heads and pronota snipped off with sharp scissors, any internal organs remaining attached to the head were removed with fine forceps, as was any residual food material in the buccal cavity. The legs and wings were removed from the bodies which were then slit ventrally and the internal organs removed. The various parts were weighed within 5-10 min of dissection in order to reduce to a minimum errors likely to arise from drying of the tissues, this is especially necessary in the case of the body.

Extraction of pigments Each portion was ground in a mortar with acid washed silver sand and, if necessary, a small amount of anhydrous Na_2SO_4 , the carotenoids were then extracted with cold acetone *in situ* by stirring carefully with a pestle. The acetone was decanted and filtered through a G4 sintered glass filter. The residue was re-extracted with acetone, and the process repeated until no colour showed in the acetone, three extractions were generally sufficient.

Chromatographic separation of the carotenoids The acetone was removed under nitrogen, and the residue immediately dissolved in a small volume (5-10 ml.) of light petroleum (b.p. 40-60°). This solution was run through a small column (10 x 2 cm) of defatted bone meal (Mann, 1943; Goodwin & Morton, 1946; Glover, Goodwin & Morton, 1948) and the column washed with further small quantities of light petroleum until no more pigment was eluted, this fraction consisted of β carotene only. The astaxanthin, sometimes contaminated by traces of carotenoid oxidation products, was then eluted with acetone. The volumes of the β carotene and astaxanthin fractions were then suitably adjusted for spectrophotometric analysis.

Spectrophotometric analysis Measurements of light absorption were made in a Beckman quartz photoelectric spectrophotometer. The measurements of both solutions (β carotene and astaxanthin) were made at 450 m μ . The wavelength 450 m μ . was used for both solutions in order to

reduce the time required to examine a considerable number of solutions. This wavelength is λ_{max} for β carotene but not for astaxanthin, however, the $E_{1\text{ cm}}^{1\%}$ 450 m μ . for pure astaxanthin in acetone is known (*vide infra*).

Calculation of results The $E_{1\text{ cm}}^{1\%}$ 450 m μ . values for the carotene and astaxanthin fractions for the various parts of the body were calculated using the expression $E_{1\text{ cm}}^{1\%} = E/cd$, where E is the observed extinction for a solution of $c\%$ in a cell d cm thick. In order to obtain an accurate $E_{1\text{ cm}}^{1\%}$ value for the whole insect a weighted mean value of $E_{1\text{ cm}}^{1\%}$ was calculated, e.g.

	Wt of part (g)	$E_{1\text{ cm}}^{1\%}$	$E_{1\text{ cm}}^{1\%} \times \text{wt}$
Body	0.608	0.0233	0.01417
Head and pronotum	0.210	0.0193	0.00405
Legs	0.234	0.0178	0.00416
Total	1.052		0.02238

$$\text{Weighted mean } E_{1\text{ cm}}^{1\%} = \frac{\sum (E_{1\text{ cm}}^{1\%} \times \text{wt.})}{\sum \text{wt.}} = \frac{0.02238}{1.052} = 0.0213$$

The amount of carotene or astaxanthin per insect can then be calculated using the expression

$$\text{Amount } (\mu\text{g}) = \frac{E_{1\text{ cm}}^{1\%} \text{ 450 m}\mu \text{ (obs)} \times \text{wt} \times 10^6}{E_{1\text{ cm}}^{1\%} \text{ 450 m}\mu \text{ of pure pigment}}$$

$E_{1\text{ cm}}^{1\%}$ 450 m μ . for pure β carotene in light petroleum was taken as 2500 and for astaxanthin in acetone, 1983. This latter figure was obtained from the curve in acetone of spectroscopically pure astaxanthin (Goodwin & Srisukh, 1949) assuming that $E_{1\text{ cm}}^{1\%}$ (λ_{max}) in acetone is the same as that in cyclohexane, i.e. 2500 (Karrer & Würgler, 1943).

RESULTS

A very considerable mass of data has been accumulated and analysed, but in order to conserve space and simplify presentation only summarizing tables are presented. An important conclusion, which allowed the Tables to be considerably simplified, was reached when the complete data were considered. It was that there are no significant differences in the concentrations of the pigments in the body, head and pronotum, and legs, and that the relative amounts of pigment in these tissues (about 50, 25-30, and 20-25%, respectively, of the total) merely reflect the relative weights of these parts. Because of this it has been considered unnecessary to record the results of analyses on the separate parts of the body, and only those referring to whole insects are presented and discussed.

In Tables 1-4 are collected the values for both concentrations and amounts of carotene and astaxanthin of whole insects at most stages of development of solitary and gregarious *Locusta* and *Schistocerca*.

The most important point which emerged from a study of these tables is that in both *Locusta* and

Table 1 *Carotenoid distribution in gregarious Locusta*

Stage	No of insects examined	No of batches examined	β Carotene		Astaxanthin	
			$E_{1\text{ cm.}}^{1\%}$ 450 m μ . (petrol)	Amount/ insect (μg)	$E_{1\text{ cm.}}^{1\%}$ 450 m μ . (acetone)	Amount/ insect (μg)
First	250	6	0 018	0 13	0 028	0 21
Second	105	4	0 023	0 38	0 052	0 97
Third	63	3	0 015	0 40	0 037	1 4
Fourth	34	3	0 028	2 1	0 021	1 8
Fifth	32	5	0 018	3 0	0 026	5 5
Immature (male)	19	7	0 029	8 4	0 0078	3 5
Immature (female)	27	7	0 027	10 7	0 0061	3 0
Mature (male)	35	9	0 029	15 4	0 0050	3 4
Mature (female)	30	9	0 036	35 8	0 0045	5 4
Immature (female) (blue)	4	4	0 0061	1 8	0 413	15 5
Mature (male) with protozoan infection	4	2	0 015	9 1	0 0077	5 8
Mature (female) with protozoan infection	7	6	0 0072	7 4	0 011	15 0

Table 2 *Carotenoid distribution in solitary Locusta*

Stage	No of insects examined	No of batches examined	β Carotene		Astaxanthin	
			$E_{1\text{ cm.}}^{1\%}$ 450 m μ . (petrol)	Amount/ insect (μg)	$E_{1\text{ cm.}}^{1\%}$ 450 m μ . (acetone)	Amount/ insect (μg)
First	46	2	—	—	0 029	0 22
Fourth	4	1	—	—	0 044	2 9
Fifth	7	2	—	—	0 030	5 0
Immature (male and female)	4	4	0 039	11 4	0 016	5 8
Mature (male)	3	3	0 035	19 1	0 010	7 1
Mature (female)	2	2	0 026	12 5	0 021	12 6

Table 3 *Carotenoid distribution in gregarious Schistocerca*

Stage	No of insects examined	No of batches examined	β Carotene		Astaxanthin	
			$E_{1\text{ cm.}}^{1\%}$ 450 m μ . (petrol)	Amount/ insect (μg)	$E_{1\text{ cm.}}^{1\%}$ 450 m μ . (acetone)	Amount/ insect (μg)
First	56	2	0 001	0 026	0 044	0 13
Second	36	3	0 031	0 63	0 34	0 72
Third	44	4	0 035	1 7	0 026	1 6
Fourth	22	3	0 016	1 7	0 028	4 1
Fifth	5	2	0 026	8 7	0 024	7 6
Immature (male)	12	5	0 038	22 9	0 016	12 5
Immature (female)	11	5	0 028	13 6	0 013	8 3
Mature (male)	6	3	0 069	44 0	0 0043	3 5
Mature (female)	2	1	0 061	55 8	0 014	16 5

Table 4 *Carotenoid distribution in solitary Schistocerca*

Stage	No of insects examined	No of batches examined	β Carotene		Astaxanthin	
			$E_{1\text{ cm.}}^{1\%}$ 450 m μ . (petrol)	Amount/ insect (μg)	$E_{1\text{ cm.}}^{1\%}$ 450 m μ . (acetone)	Amount/ insect (μg)
First	42	1	—	—	0 039	0 39
Fourth	3	1	0 042	5 5	0 053	5 6
Fifth	3	1	0 046	26 4	0 023	17 7
Immature (male and female)	5	3	0 045	25 3	0 014	10 1
Mature (male and female)	3	2	0 064	47 5	0 017	16 2

Schistocerca there is no difference in respect of carotenoid distribution between the solitary and gregarious phases, further, there is no difference between *Locusta* and *Schistocerca* in the concentrations of the pigments, but there is a tendency for adult *Schistocerca* to contain more of the pigments

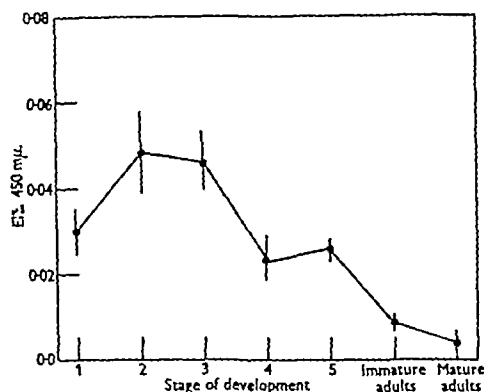


Fig 1 The changes in the astaxanthin concentration ($E_{1\text{ cm}}^{1\%} 450 \text{ m}\mu$) of both phases of *Locusta* and *Schistocerca* during development. The vertical lines indicate the standard deviation of the results

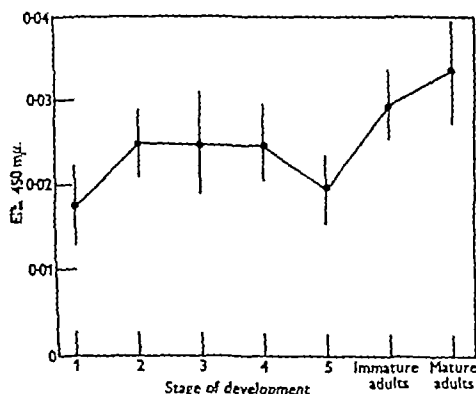


Fig 2 The changes in the carotene concentration ($E_{1\text{ cm}}^{1\%} 450 \text{ m}\mu$) of both phases of *Locusta* and *Schistocerca* during development. The vertical lines indicate the standard deviation of the results

than do adult *Locusta*, this is again merely a reflexion of the weight variations between the species. There is, however, a marked and important difference in the storage of the two pigments during development. As this difference obtains in both species, irrespective of phase, 'grand means' of the concentrations (measured as $E_{1\text{ cm}}^{1\%}$) and amounts of the two pigments have been calculated for each stage and plotted in Figs 1-3.

Considering concentrations first, it will be seen (Fig 1) that the astaxanthin concentration remains reasonably constant from birth to the third hopper stage, and thereafter diminishes until in mature

adults it is not more than one quarter to one third of that of the early hopper values. With carotene the situation is different (Fig 2), the carotene concentration at birth increases somewhat as soon as the insects begin to eat and then remains near a constant value until the insects become adult when the concentration increases until at maturity it is at least twice that at birth.

This difference between carotene and astaxanthin is reflected in the amount of the pigments present in the developing insects (Fig 3). During the hopper stages the astaxanthin content increases gradually but rather slowly, the increase ceases at adult

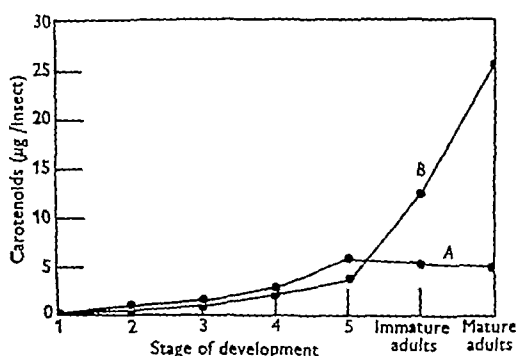


Fig 3 The changes in the carotenoid content of locusts during post-embryonic development. A, astaxanthin content, B, β carotene content

emergence, and throughout adult life the levels remain constant at or just below the fifth stage levels. The increase in β carotene content in general parallels that of astaxanthin until the insects become adult when, in contrast to astaxanthin, it increases during adult life, this is especially marked after sexual maturity when considerable stores are found in the fatty tissues.

Carotenoids in the eggs Newly laid eggs of both *Schistocerca* (Chauvin, 1941b, Goodwin & Srisukh, 1948, 1949) and *Locusta* (Goodwin & Srisukh, 1948, 1949) contain β carotene only. In Fig 4 are recorded the results of an investigation into the quantitative and qualitative changes in the carotenoid content of the developing *Locusta* egg. It will be seen that apparently β carotene takes no part in the process of embryonic development until near the end of the period. It is only on about the 7th day that it begins to disappear and astaxanthin to appear, both these processes then continue until the insect is hatched. A similar experiment using *Schistocerca* eggs yielded very similar results, and is not recorded here.

It will be noted that the average carotene content of a *Locusta* egg is $0.38 \mu\text{g}$. This means that an average egg pod of 50 eggs will contain about $20 \mu\text{g}$ of β carotene, the corresponding mean carotene level in *Schistocerca* eggs is about $1.0 \mu\text{g/egg}$.

Carotenoids in exuviae The appearance of carotenoids in the exuviae of any hopper stage has never been detected

Carotenoids in dark blue Locusta During the course of this investigation four atypical immature females were examined, they were very dark blue, had a metallic lustre, and were progeny of normal parents. However, it was noted that the subcutaneous fatty material was completely colourless, although one would have expected it to have been bright yellow owing to the presence of stored β -carotene. Chromatography of the carotenoid extract showed that the carotenoid present was almost completely astaxanthin, β carotene was detected, but only in traces (see Table 1). The astaxanthin levels were much higher than normal.

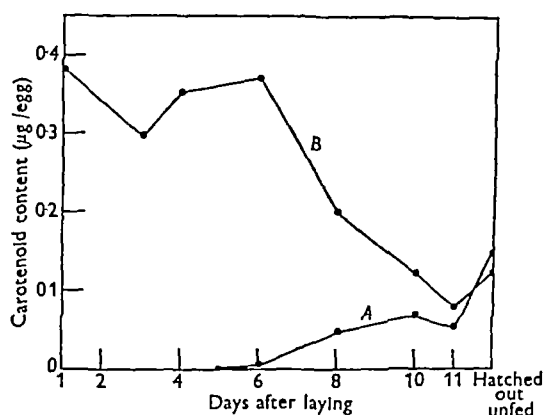


Fig 4 The changes in the carotenoid content of fertilized *Locusta* eggs during embryonic development. A, astaxanthin content, B, β carotene content

Carotenoids in infected insects It has been possible to examine locusts infected with a protozoan, probably *Phlebotomus*. The infected insects have a normal external appearance except that the outside of the abdomen becomes a characteristic pink. When they were opened it was found that the fatty tissues had lost their yellow colour and, except for the occurrence of pink spots, were almost white, it was therefore no surprise to find that, compared with healthy insects, the carotene contents of both males and females were very low, this was most marked in the case of heavily infected insects, especially females. On the other hand, the astaxanthin contents of both sexes were normal (Table 1). It was noted that the developing eggs of infected females still contained carotene, but it was not possible to ascertain the amount.

DISCUSSION

Chauvin (1941b) stated that the pink carotenoid recently identified as astaxanthin (Goodwin & Srisukh, 1948, 1949) is characteristic of immature locusts and that the yellow carotenoid (β carotene)

is characteristic of adults. This generalization can now be seen to have only a limited validity, but it did suggest the existence of an important difference between the metabolism of β carotene and astaxanthin in hoppers and adult locusts. This difference has now been quantitatively demonstrated. At birth about 70% of the total carotenoids is astaxanthin, this value gradually decreases until the fifth hopper stage, however, when the locusts become adult the accumulation of β carotene proceeds at an enhanced rate whilst the amount of astaxanthin present remains stationary. This results in astaxanthin being never more, and often much less, than 30% of the total carotenoids in adult insects.

It is not known why astaxanthin storage ceases when locusts become adult. It may be due to the fact that the rate of synthesis is reduced or stopped altogether, or that synthesis continues at the same rate, but that adult locusts utilize (or destroy) the pigment to a greater extent than do hoppers. It is interesting to note that wings, which are fully formed only in adults, contain relatively more astaxanthin than carotene, however, as they contain relatively little pigment compared with the rest of the body (Goodwin & Srisukh, 1949) it is not possible to postulate that astaxanthin synthesis in adults is diverted to the wings rather than to the body.

Formation of astaxanthin This pigment, which is not present in the food of the locust, must be produced in one of three ways: (a) *de novo*, (b) by oxidation of alimentary carotenes, or (c) by oxidation of alimentary xanthophylls. The first possibility is almost certainly ruled out, for a survey of carotenoid biochemistry reveals no authenticated instance of carotenoid synthesis in animals (Goodwin, 1949a). Up to the present it has not been possible to decide which (if not both) of the other alternatives operates, for, as no adequate artificial diet is yet available, it has not been possible to carry out feeding experiments. No plant xanthophylls are ever found in locusts outside the alimentary tract, so that these pigments might either be converted quantitatively into astaxanthin or be quantitatively excreted, the latter possibility was put to experimental test. The relative amounts of carotenoids and xanthophylls were determined in *Locusta* faeces collected at all stages of development, together with the grass upon which they were feeding. It was considered that if only xanthophylls were found in the faeces or if the faecal xanthophyll/carotene ratio was very much higher than that of grass then it would be strong presumptive evidence that xanthophylls were preferentially excreted. However, it was found that locust faeces always contained a considerable amount of undigested or partly digested grass and were thus always very rich in both xanthophylls and carotenes, which occurred in ratios not differing significantly from those of the ingested grass. It was

therefore impossible to reach any conclusions as to the ability of the locust to absorb xanthophylls

There is one fact which favours the suggestion that carotene is the precursor of astaxanthin. Astaxanthin is formed during the incubation of the egg, and, as it is unlikely to be produced *de novo*, carotene appears to be the only possible precursor

Carotenoids in the developing egg It has been shown quite clearly that β carotene is used up during the later stages of embryonic development and that astaxanthin is formed. As has just been suggested, it is likely but not certain that astaxanthin is formed by the oxidation of β -carotene, even if this were so the astaxanthin produced does not account for all the β carotene which disappears. What function the β carotene plays in the developing locust egg is not known. Although carotenoids are often preferentially stored in the eggs of lower animals, little is known of their function, and it appears that the present work is the first to demonstrate that β -carotene is actively metabolized during embryonic development of the egg. In contrast, it should be noted that Mann (1946) and Suomalainen (1939) have reported no loss of carotenoids during the development of hens' eggs.

Any function in locust eggs which may eventually be assigned to carotenoids is perhaps unlikely to be a general one, for a number of insects, amongst which is the orthopteran *Blattella germanica* (McCay, 1938, Bowers & McCay, 1940), can exist on a diet free from carotene or vitamin A.

As has been stated earlier, the amount of carotene in a *Locusta* egg pod, containing, on the average, 50 eggs, can amount to at least 20 μg . This is of the same order as the amount found in a female without eggs and in an adult male. It seems, then, that females absorb more carotene, perhaps by eating more food, to provide the required amount for their eggs, for these obviously do not obtain their carotene at the expense of the other body tissues. The results of a limited number of experiments indicate that *Locusta* and *Schistocerca* eggs contain respectively about 0.4 and 1.0 μg of β carotene/egg. This difference is due mainly to the difference in size, for the weight of a *Schistocerca* egg is about 0.01 g whilst that of a *Locusta* egg is about 0.06 g. The concentration of carotene in *Locusta* and *Schistocerca* eggs, therefore, does not differ significantly, and is about 100 $\mu\text{g/g}$ of eggs (wet weight), this is of the same order but somewhat higher than the value for *Schistocerca* eggs reported by Brodskis (1944) and Brodskis & Rungs (1944) who obtained 95 $\mu\text{g/g}$ dry weight, assuming a value of 30% for the dry weight of the eggs, this reduces to about 30 $\mu\text{g/g}$ wet weight. On the other hand, the carotene content of grasshopper (*Melanoplus bivittatus* Say) eggs has been reported to be 246 $\mu\text{g/g}$ dry weight (Grayson, 1942, Grayson & Tauber, 1943).

Relationship between carotenoids and colour of locusts It has already been stated that there are no significant differences between the carotenoid contents of gregarious and solitary locusts, but it will be interesting to consider the other important stage of colour differentiation, that accompanying the onset of sexual maturity. At this stage males become yellow and females dark brown. It has been established in the case of *Locusta*, at least, that acridioxanthin, the main pigment of locusts other than the carotenoids, is unchanged in amount, state of oxidation and position in the integument* (Goodwin, 1949b) in males and females. The experiments reported here (see Table 1) have shown that the concentration of carotene in both sexes is the same, the amounts in the females are somewhat greater owing to their greater size. It seems reasonable to assume that the yellow colour which the males assume when they reach maturity is due to a migration of part of their carotenoids to the outer layers of the cuticle, for acetone will dissolve out a considerable amount of carotene from a piece of yellow cuticle carefully freed from fat. Owing to technical difficulties histological confirmation of this has not yet been achieved, but it is interesting to note in this respect that in males infected with *Phthorophora* (Table 1) the loss of carotene is less than in infected females. This could be explained by assuming that the cuticle carotene in the males was only slightly affected compared with that stored in the adipose tissues, a similar survival of 'functional' carotene was noted in the eggs of infected females. Chauvin (1941b) has previously claimed that the yellow coloration of mature *Schistocerca* is due to β carotene.

Although the striking colour differences between solitary and gregarious phases cannot be due to differences in the carotenoid content of the insects as a whole, it is very likely that they are due to differences in carotenoid distribution especially near the cuticle.

Carotenoid distribution in blue locusts In the atypical immature female *Locusta* the acridioxanthin content was normal (Goodwin, 1949b), but instead of containing a mixture of carotene and astaxanthin, the former preponderating, only traces of carotene were present, but astaxanthin was present in excess (Table 1), further, astaxanthin must have been present in the integument, for the fatty tissues of the body were colourless. It may be that the outstanding blue colour of these locusts was due to an integumentary astaxanthin protein, such as occurs in the purple patches of lobster carapaces (Kuhn & Lederer, 1933). Such a (brown) astaxanthin complex has been shown to occur in the wings of normal locusts, but up to now has not been found in their

* The author is grateful to Dr R. J. Daniel, Department of Oceanography, University of Liverpool, for the histological examinations of the locust integument.

integuments (Goodwin & Srisukh, 1949) It is possible that these insects were mutants in which an enzyme was produced (or activated) which either converts β carotene very efficiently into astaxanthin or oxidatively destroys it Such genetic control of carotenoid metabolism in insects has been noted previously (Gerould, 1921, Uda, 1919, Przibram & Lederer, 1933)

The value of locusts as a nutritional source of carotene The nutritional importance of locusts, especially in North Africa, cannot be overlooked (Grant, 1948), as the carotene content of a gutted, mature locust is at least $30 \mu\text{g}$, the value for an intact animal will be higher owing to the presence of carotene in the alimentary tract Our investigations on whole *Locusta* indicate that their carotene content varies from $35 \mu\text{g}$ for mature males to $70 \mu\text{g}$ for mature females with eggs An average value for mature *Schistocerca* has been given (Brodskis & Rungs, 1944) as $256 \mu\text{g/g}$ dry weight which, assuming 70% of water, reduces to $80 \mu\text{g/g}$ wet weight and to about $120 \mu\text{g/insect}$ It will be seen that locusts are as good a source of β carotene as are most green vegetables In fact, it is quite possible that humans would utilize 'locust carotene' rather better than 'plant carotene', for the locust pigment is associated with considerable amounts of fat and it is well known that the presence of fat generally improves carotene absorption (see e.g. Hume & Krebs, 1949) It is interesting to note that from the carotene viewpoint the higher price charged for well developed females (Dubois, 1893) is justified

SUMMARY

1 The distribution of β carotene and astaxanthin has been studied in the solitary and gregarious phases of two locust species *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria*

Forsk The locusts were examined at most stages of development, and the relative distribution in body, head and pronotum, and legs was also studied

2 In both species no significant differences in the carotenoid distribution in the three main body regions occur between solitary and gregarious insects, further, no differences were noted between the two species

3 The relative amounts of astaxanthin and carotene alter during development In the early hopper stages astaxanthin comprises about 70% of the total carotenoids, whilst in mature insects this drops to below 30%

4 When the β carotene content of the eggs is allowed for, no differences are found in the carotenoid concentration of male and female insects

5 No carotenoids were detected in locust exuviae

6 Newly laid *Locusta* and *Schistocerca* eggs contain β carotene to the extent of about $100 \mu\text{g/g}$ (wet weight) During the later stages of pre-embryonic development β -carotene disappears and astaxanthin is formed

6 In four atypical *Locusta*, which were very dark blue, astaxanthin occurred in considerably increased amount and carotene only in traces

7 *Locusta* infected with the protozoan *Phistophora* store very little carotene but normal amounts of astaxanthin

8 The possible importance of locusts, which contain β carotene (provitamin A) in amounts similar to those found in green vegetables, as a source of vitamin A in some native dietaries is indicated

The author is indebted to the Anti Locust Research Centre for material and a grant towards expenses, the help and advice of Drs B. P. Uvarov and D. L. Gunn of this centre have been invaluable Thanks are also due to Prof R. A. Morton not only for his kindly interest but also for making available facilities and apparatus

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The Enzymic Degradation of Adenosinetriphosphate

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(Received 16 May 1949)

The present investigation arose from the observation that samples of adenosinetriphosphate (ATP), freshly prepared from skeletal muscle, gave a satisfactory phosphate cleavage with myosin adenosinetriphosphatase, whilst samples stored as the barium salt at room temperatures were very deficient in this respect. By the use of a specific inorganic pyrophosphatase (Bailey & Webb, 1944) it was later shown (Bailey & Webb, unpublished) that such specimens contained as much as 12% of the labile phosphorus as inorganic pyrophosphate, indicating a breakdown of ATP to this latter substance and adenylic acid. It may be recalled that Lohmann (1928), before his characterization of ATP, considered the labile phosphorus of muscle to be inorganic pyrophosphate, and did in fact isolate the crystalline sodium salt. Later, he considered it to have arisen during the preparative procedure (Lohmann, 1931).

It is clear that ATP preparations decomposing in this way may show a satisfactory ratio of total phosphorus/labile phosphorus/total nitrogen, and other criteria of purity must be employed. For this purpose enzyme methods have been explored. The purity both of laboratory and commercial specimens of ATP has been assessed, and changes occurring during known conditions of storage have been followed.

EXPERIMENTAL

Enzyme preparations

Myosin adenosinetriphosphatase. Myosin was prepared in the conventional manner from rabbit muscle (see Bailey, 1942), and was five times precipitated from water. It was stored in 0.5M KCl at pH 7 and 0° in presence of a trace of toluene, and was efficient enzymically over a period of 3-4 weeks.

Myokinase. The method of preparation followed strictly that of Colowick & Kalckar (1943). The enzyme was stored

at 0° as an ammonium sulphate filter cake, and dissolved in dilute veronal buffer (pH 7) before use. As a paste, the enzyme remains active indefinitely.

Yeast inorganic pyrophosphatase. The purified enzyme prepared by Bailey & Webb (1944) was found to be active after storing at 0° for 5 years. In the initial experiments, a new batch of crude enzyme was prepared from brewer's yeast. It was found that yeast autolysate, after dialysis, contained not only the very active pyrophosphatase, but also a weak, Mg activated adenosinetriphosphatase. After submitting the autolysate to the first four stages of the fractionation procedure of Bailey & Webb (1944), this latter enzyme had disappeared, leaving a very active pyrophosphatase suitable for the present work. Although the original enzyme has been used throughout, the preparation of new enzyme does not require the laborious procedure first employed. Preparations must be stored as an ammonium sulphate protein paste at 0°.

Potato apyrase. This enzyme, discovered by Berger, Colowick & Slein (unpublished, see Kalckar, 1944a), splits off two phosphate groups from ATP. In critical experiments, however, it was found that preparations made according to the directions of Kalckar (1944a) are feebly active with respect both to inorganic pyrophosphate and adenylic acid. Our findings are supported in a recent paper by Krishnan (1949), who has compared the substrate specificity of crude and purified enzyme. In high concentration, even the purified enzyme possesses nucleotidase activity, but not at concentrations which are still able to split off two phosphate groups from ATP. It would appear, therefore, that potato apyrase is mixed with other phosphatases, and the usefulness of this enzyme is somewhat limited.

Incubation conditions

General. After establishing the complete specificity of the enzyme preparations, three test systems were adopted: the splitting of ATP (a) by myosin adenosinetriphosphatase, (b) by myosin adenosinetriphosphatase in presence of myokinase, and (c) by yeast inorganic pyrophosphatase. Additional experiments with potato apyrase were sometimes carried out. In (a), only the terminal phosphate group is split, whilst in presence of myokinase the adenosinedi-

phosphate (ADP) formed dismutates to adenylic acid and ATP, so that in the limit, the whole of the ATP is converted to adenylic acid. With pure ATP, the phosphate split by (a) should be half that of (b), but if ADP is present initially as impurity, (b) > 2 (a). Supposing also that inosinetriphosphate (ITP) is present, then, in absence of ADP impurity, (b) < 2 (a), since ITP is degraded to inosinediphosphate (IDP) by adenosinetriphosphatase, but IDP is not attacked by myokinase (Kleinzeller, 1942). Indirect evidence for inorganic pyrophosphate impurity is also obtained if 2 (a) or (b) fall far short of the values calculated from the labile P.

Without exception, tests were carried out in presence of excess enzyme, and the hydrolysis followed with a constant amount of enzyme over varying periods of time. Temperatures did not exceed 25° to avoid inactivation (see below), but this possibility was always assumed, and additional enzyme was added to several tubes of a series towards the end of the incubation period.

Myosin and myokinase A tube series must be used rather than a single incubation mixture, since the splitting of phosphate in presence of Ca activator gives rise to the progressive precipitation of Ca phosphate, and accurate sampling is impossible. Each tube contained 1 ml 0.5M glycine buffer (pH 8.6 at 20°), 0.1 ml 0.1M CaCl₂, 0.1 ml myosin solution (0.5 mg protein), Na ATP solution (0.2 mg labile P), diluted with water to 1.6 ml. Another series was set up simultaneously, containing, in addition, myokinase (equivalent to 5 mg protein paste). The tubes were incubated at 25° and the reaction arrested by adding 0.5 ml of 10% (w/v) trichloroacetic acid. After standing at least 1 hr in ice water, the protein was filtered off through small plugs of cotton wool into 25 ml standard flasks, and the tubes washed with two 1 ml portions of 3% trichloroacetic acid. The free phosphate, here and below, was measured colorimetrically in a photoelectric colorimeter by the Fiske Subbarow method.

Yeast pyrophosphatase and potato apyrase The incubation conditions for the former have already been described (Bailey & Webb, 1944). For the potato enzyme, 0.05 ml 0.1M CaCl₂ was added to 2 ml 0.03M veronal (pH 7), followed by enzyme and ATP to a volume of 2.5 ml. With both enzymes deproteinization was unnecessary, and the reaction was arrested by addition of trichloroacetic acid to pH 1.

Adenosinetriphosphate preparations

Preparation The general procedure followed that of Lohmann as described by Needham (1942). The dibarium salt, without drying, was acidified to pH 1 with HCl, separated from any insoluble residue, and precipitated as the monobarium salt with an equal volume of ethanol (cf. Kerr, 1941a). The dibarium salt was again prepared by dissolving the monobarium salt in water and neutralizing with NaOH in presence of a slight excess of Ba acetate. After centrifuging, it was converted to the monobarium salt, which was finally dried in ethanol, ether, and *in vacuo* over night. All preparations were stored at 0°.

Decomposition When Ba⁺⁺ is removed as sulphate in the manner previously described (Bailey, 1942), there is a loss of 20% of the ATP which is adsorbed on the BaSO₄. This loss may be minimized in the following way. Approximately half the Ba⁺⁺ is removed as sulphate at pH 0.5–1, and the resulting solution run through a column of prepared Amberlite IR-100 (see Polak & Meyerhof, 1947; Kelly & Meyerhof, 1948). A solution equivalent to 0.2–0.3 g of

Ba ATP can be employed for a 20 cm column containing 3 g of resin. The effluent emerges at pH 4.5, and is further neutralized to pH 6.5. (The resin is first activated by soaking in 5N HCl overnight, washed with water, then with 4% Na₂CO₃, and finally with water, the column may be recovered thereafter by washing with 2N HCl.) The removal of Fe⁺⁺⁺ in ATP preparations by resin is only partial, and in this respect an acid column is more efficient than that described.

RESULTS

The labile phosphorus of adenosinetriphosphate Following the procedure of Lohmann (1931) most workers have determined the labile P by hydrolysing in N-HCl for 7 min at 100°. Both Fiske (1934) and Kerr (1941a), on the other hand, recommend a

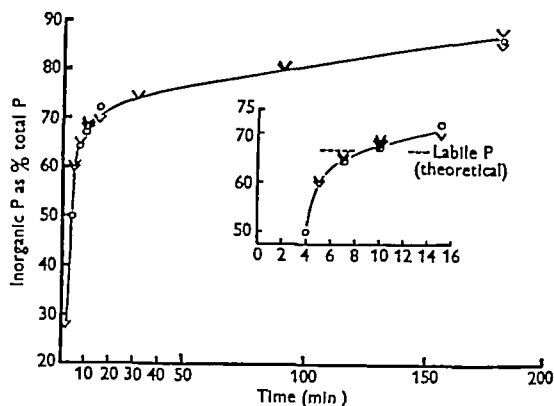


Fig 1 Hydrolysis of ATP in N-HCl (boiling water bath, 1 ml. samples) Inset part of curve enlarged. v, preparation 1 (3 experiments), O preparation 2 (1 experiment) Both samples prepared by author

15 min period. Whilst the shorter period of 7 min seems just sufficient for inorganic pyrophosphate, it gives values low by about 3% in the case of ATP (1 ml samples). If the degradation of ATP by acid occurs partly at the terminal P group and partly at the penultimate, the ADP and inorganic pyrophosphate thus formed will themselves follow a unimolecular course of hydrolysis which will be complete in some period > 7 min. The time at which labile P is just two thirds of the total P will occur at a point where the P remaining in pyrophosphate linkage equals the phosphate contributed by the breakdown of adenylic acid (more strictly, of ribose 5 phosphate). This point, according to present findings (Fig 1), is 9–10 min after immersion of 1 ml samples in boiling water.

Degradation of adenosinetriphosphate by myosin and myokinase Fig 2 illustrates the results of a typical experiment with a laboratory ATP preparation. A characteristic feature is the rapid initial splitting by adenosinetriphosphatase of 40% of the labile P (theory 50%), and thereafter a slow decomposition to the final value. The latter phase could

represent (a) a low rate of hydrolysis at low substrate concentration, (b) inhibition by ADP, (c) inhibition by phosphate. The points lying near curve A, Fig 2, show that ATP in low concentration, either alone or in presence of a large excess of ADP, is rapidly split, but that in presence of inorganic phosphate, the breakdown is relatively slow. The inhibition by free phosphate may be more apparent than real, since in the later stages of the reaction Ca phosphate is precipitated. The facility with which

phosphorylation of glucose by yeast hexokinase, were found to contain inorganic pyrophosphate (Bailey & Webb, unpublished). The unique affinity of pyrophosphatase for its substrate gives a progress curve which is linear almost to the point where substrate is exhausted (Bailey & Webb, 1944). This effect is shown again in Fig 3 where progress curves

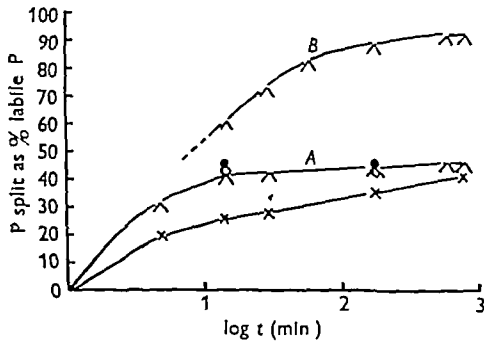


Fig 2 Hydrolysis of a fresh specimen of laboratory ATP by enzymes A, adenosinetriphosphatase, B, adenosmetri phosphatase myokinase, each curve represents hydrolysis of ATP equiv to 0.17 mg labile P. ●, 0.05 mg labile P of ATP without additions, ○, 0.05 mg labile P of ATP + 0.23 mg labile P of ADP, ×—×, 0.024 mg labile P of ATP + 0.23 mg inorganic phosphate, all in presence of adenosmetriphosphatase alone

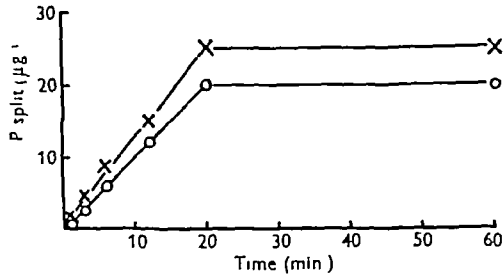


Fig 3 Hydrolysis of morganic pyrophosphate by yeast pyrophosphatase ×—×, $\text{Na}_2\text{P}_2\text{O}_7$, ○—○, pyro phosphate in a stored commercial ATP preparation

nucleotides form double salts makes it possible that some ATP is adsorbed by this precipitate and is less accessible to the enzyme. The addition of inorganic pyrophosphate in amounts sometimes found in ATP preparations (see below) has no significant effect upon the initial or final rates of splitting.

After 10 hr at 25°, the splitting in the presence of myosin and myokinase (curve B, Fig 2) is 92% of the labile P, and that with myosin alone (curve A), 47%. After this period, the enzymes are still capable of splitting added ATP, so that the cessation of hydrolysis must be due either to the attainment of an equilibrium, apparent or real, or to the presence of impurities. These points are more appropriately discussed below.

Inorganic pyrophosphate in adenosmetriphosphate preparations. Referring to his previous isolation of sodium pyrophosphate from muscle, Lohmann (1931) comments: 'The former investigations were in error, through the spontaneous decomposition of this compound (ATP) as the Ba salt in neutral or weakly alkaline solutions.' This warning as to the instability of ATP seems generally to have been ignored. When tested with yeast pyrophosphatase, old preparations of ATP which contained labile phosphate unavailable either to myosin or for the

are given for pyrophosphate comparable in concentration to that found in a commercial ATP preparation which had been stored at room temperature for an unknown period. The absolute specificity of the enzyme, and the similar slopes, make it certain that the component degraded is inorganic pyrophosphate.

Table 1 Enzymic assay of inorganic pyrophosphate produced during storage of adenosmetriphosphate samples

ATP sample	Date of testing	Inorganic pyro phosphate (% of labile P)	Rate of breakdown (% of labile P/month)
Commercial (monocalcium salt)	1 Feb	1	
	21 Sept	12	1.5
Commercial (monobarium salt)	15 Jan	2.5	
	4 May	5	0.7
Commercial (dibarium salt)	16 Jan	2	
	21 Sept	7.5	0.7
Commercial (monobarium salt)	26 Jan	8	
	27 Nov	19	1.1
Laboratory (monobarium salt)	26 Jan	1	
	7 May	5	1.2
Laboratory (monobarium salt)	10 July	Nil	
	9 Sept	1.5	0.8
Laboratory (solution of Na salt at -10°)	Dec 1947	Nil	
	June 1948	Nil	Nil
Laboratory ADP (solution as above)	Dec 1947	?	
	Oct 1948	2.5	'
Mean for Ca and Ba salts			1.0

Both monobarium and dibarium salts, as also the Ca salt, break down progressively to pyrophosphate and adenylic acid on storage, even at 0°. Table 1 summarizes the pyrophosphate content of various

ATP preparations after storing at 0°. As a percentage of the labile P, the rate of production of inorganic pyrophosphate/month varies between the extremes of 0.7 and 1.5%, mean 1.0%. Whether the water content of the preparations influences breakdown has not been determined. In contrast, no pyrophosphate has been detected after storing solutions of Na ATP at pH 6.5 in the frozen state even after 7 months, and only 2.6% in a preparation of ADP similarly stored (this may have been present originally).

Analysis of some adenosinetriphosphate preparations. In Table 2 are collected chemical and enzymic analyses of laboratory and commercial ATP preparations. The ratio total P/labile P (each corrected

The labile P accounted for enzymically is never more than 93–95%, even in laboratory preparations. Commercial preparations generally contain more free P and inorganic pyrophosphate, and show a lower rate of splitting with myosin adenosinetriphosphatase. To check whether the enzyme assay gives a true measure of purity, the end products were isolated and again subjected to enzyme action. The assay by the muscle enzymes was also compared with the action of potato apyrase.

The action of potato apyrase on adenosinetriphosphate. Kalekar (1944a) records that the terminal group of ATP is split faster than that of ADP. The progress curve, given in Fig. 4, consists of two portions, the first representing mainly the splitting of

Table 2 *Chemical and enzymic analysis of commercial and laboratory preparations of adenosinetriphosphate*

Source	Type of salt	Total P (%)	Chemical analysis as percentage of total P*			Enzymic analysis as percentage of labile (10 min.) P		
			Labile P	Free P	Ratio total P/labile P	ATPase† + myokinase	Pyrophosphatase (yeast)	
Author (fresh sample‡)	Monobarium	11.95	7.85	0.05	1.52	44.5	92	1
As above (stored 3 months)		—	—	—	—	43	86	5
Author (stored 6 weeks)	Monobarium	11.0	7.55	0.14	1.46	41.5	91.5	1.5
E.J.M.	Monobarium	—	—	—	—	46	92	1.5
Commercial (stored 1 yr)	Monobarium	11.67	8.25	0.8	1.41	34.5	71	19
Commercial (fresh)	Dibarium	8.95	5.75	1.3	1.55 (1.45)	43	87	2
As above, stored 4 months		—	—	—	—	38	82	7.5
Author (stored 2 yr at room temp.)	Dibarium	9.65	5.4	2.5	1.79 (1.53)	18	51.5	41
Advanced class (stored 5 yr at room temp.)	Dibarium	7.76	4.72	1.55	1.64 (1.48)	Nil	2 (?)	95.5

* Total P and labile P are corrected for inorganic phosphate, figures in parentheses are derived from total P and labile P not corrected for inorganic phosphate (see text).

† ATPase = adenosinetriphosphatase.

‡ Atomic ratio P/N calc. 3/5, found 3/4.96.

for inorganic phosphate) is near the theoretical value of 1.5 in fresh laboratory specimens. Where the preparations have been stored, the free inorganic phosphate is high, and the ratio is only satisfactory if this latter is added both to labile and total P. This fact alone indicates some breakdown to ADP and phosphate, and the enzymic analyses support the inference, i.e. in these cases the splitting by myosin + myokinase is more than twice that with myosin alone, indicating the presence of free ADP. The breakdown of ATP to inorganic pyrophosphate on storage is thus accompanied by a less extensive decomposition to ADP and phosphate.

the terminal phosphate of ATP, and the second that of ADP. A break occurs when most of the ATP is degraded to ADP and another when hydrolysis is complete. A somewhat similar curve was obtained by Albaum & Kletzkun (1948) with ATP from *Drosophila melanogaster*. The total splitting is 7% above that obtained with myosin + myokinase, but of the 5% inorganic pyrophosphate present, 2–3% will have undergone hydrolysis by the pyrophosphatase present in the potato enzyme. Under the conditions of the experiment, the nucleotidase action can be neglected. (Initial hydrolysis rates of ATP, Na pyrophosphate and adenylic acid by apyrase are in the

ratio 1, 0.01 and 0.003 respectively) Thus, it would appear that the true ATP content is some 4 % higher than that indicated by use of the muscle enzymes. This figure is vindicated by experiments with ATP preparations containing no inorganic pyrophosphate, and by the isolation experiments below.

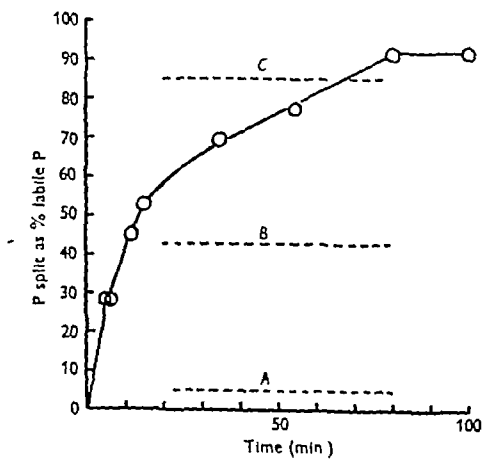


Fig 4 Hydrolysis of ATP by potato apyrase. Dotted curves: A, level of splitting with yeast pyrophosphatase; B, with adenosmetriphosphatase; C, with adenosmetriphosphatase-myokinase.

Isolation of end products after the action of myosin or myosin plus myokinase

The ATP solution is incubated for about 7 hr at 25° with myosin (or myosin + myokinase) under the standard conditions. The digest is adjusted to pH 5 with HCl, the precipitate of protein centrifuged off and the extent of hydrolysis determined on a sample of the supernatant liquid. An

excess of Ba acetate (final concentration 0.1%) is added and NaOH to a pH of 7, followed by ethanol to a final concentration of 50% (v/v). After cooling to 0°, the Ba salts are centrifuged down, suspended in water, and HNO₃ added to pH 1. The nucleotides are now precipitated in the usual manner (cf. Needham, 1942) with the Lohmann mercury reagent, decomposed with H₂S and neutralized (after aeration) with Ba acetate and NaOH (final pH 7.5). After addition of an equal volume of ethanol, the Ba salts are separated, dried in ethanol and ether, and subjected to enzymic analysis.

Table 3 summarizes the results of three experiments, two with the end products after the action of myosin alone, and one after myosin + myokinase. The recovery of labile P in the case of the first two experiments is 60–80% of the calculated amount, and most of the inorganic pyrophosphate present is recovered. The recovery of labile P from myosin-myokinase digests, on the other hand, is always low, and most of the inorganic pyrophosphate is now not precipitated with the Lohmann reagent. (This again illustrates how readily the nucleotides form double salts (cf. Kerr, 1941b).) The recovery of any undecomposed ATP is probably greater than the overall figures for the total recovery of labile P, so that the values derived in columns (b) of Table 3, corrected for isolation losses, are maximal. They indicate that the assay of ATP by the muscle enzymes is 1–2.5% low. In the best laboratory preparations, therefore, some 4–5% of the labile P has not been identified (see Table 2).

A possible impurity was at first thought to be guanylic acid, which according to Kerr & Seradarian (1945) is present in the trichloroacetic extracts of various tissues, though the amount in skeletal muscle (dog) amounts only to 3% of the adenine nucleotides. This probably arises by

Table 3 Isolation and analysis of the end products after enzymic breakdown of adenosmetriphosphate

(All values expressed as percentage of labile phosphorus)

ATP sample	Exp 1	Exp 2	Exp 3			
Method of degradation	Laboratory	Commercial	Commercial			
	ATPase*	ATPase*	ATPase*-myokinase			
Original analysis						
Split by adenosinetriphosphatase	46	33	36.5			
Split by adenosinetriphosphatase myokinase	92	69.5	80.5			
Split by pyrophosphatase (yeast)	1.5	14.5	12.0			
Analysis of large-scale digest						
Labile P split	45	33	80			
Labile P recovered as percentage of calc. value	79	61	5†			
Analysis of recovered end products ‡						
	(a)	(b)	(a)	(b)		
Split by adenosinetriphosphatase	4	2.4	2.5	1.8	2.5	1.2
Split by adenosinetriphosphatase myokinase	88	—	64	—	14.5	—
Split by pyrophosphatase	4	—	25	—	45§	—

* ATPase = adenosinetriphosphatase
† Recovery here on the basis of labile P other than inorganic pyrophosphate
‡ Values (a) as percentage of labile P present in the isolated end products (b) as percentage of original labile P of digest after correction for isolation losses
§ Of the original inorganic pyrophosphate 95% was recovered from the water soluble Hg salts

degradation of tissue nucleic acid in trichloroacetic acid (cf Kaplan & Neuberger, 1944). When ATP is hydrolysed for 10 min in N HCl, a substance is liberated which gives a blue colour with the Folin phenol reagent in alkali, though the original ATP contains no chromogenic material. If the latter were a chromogenic purine (guanine or xanthine) liberated from glycosidic linkage, the amount should remain constant after 15 min when hydrolysis is virtually complete. It was found, however, that the amount steadily increased with continued hydrolysis, and that pure adenine itself was transformed in the same way. This reaction is worthy of further study.

Control experiments had shown that under the conditions of incubation of ATP in alkaline glycine buffer, no degradation to inorganic pyrophosphate or free phosphate occurred. The possibility that some of the missing labile P had arisen by the enzymic production of pyrophosphate was also checked. In these experiments, it was necessary to acidify the myosin myokinase digests to pH 5 to inactivate adenosinetriphosphatase, to reneutralize to pH 7, add veronal buffer pH 7, and to swamp the Ca^{++} with a tenfold concentration of Mg^{++} to allow the action of pyrophosphatase, which is antagonized by Ca^{++} (Bailey & Webb, 1944). The difference in free phosphate before and after addition of pyrophosphatase was always within the experimental error. It was also shown that digests hydrolysed to the fullest extent, either by myosin or myosin myokinase, and then subjected to 10 min hydrolysis in acid, contained the amount of labile P added at the beginning of the experiment. None is lost, therefore, by transfer to a more stable form.

Adenosinediphosphate in muscle. Since ADP functions as phosphate acceptor in several reactions of the glycolytic cycle, and also as the substrate for myokinase, it seems unlikely that any appreciable

enzymic analysis difficult, whilst in (b) some ADP may be lost in the ethanol-HCl procedure used for the removal of phosphate. The presence of a small amount of ADP can, however, be shown in the mother liquors obtained after precipitating ATP.

The once precipitated Ba salt of ATP (11.9 g) was dissolved in 30 ml N -HCl, diluted with water and precipitated as the dibarium salt (total volume 1.2 l). The precipitate was spun down, dissolved in HCl (total volume 500 ml, pH 2) and an equal volume of ethanol added. The combined mother liquors were neutralized to pH 7.5, an equal volume of ethanol added, and the precipitated Ba salt dried and decomposed. Enzymic analysis gave (as percentage of the labile P) split by myosin, 23%, by myosin myokinase, 88%, by pyrophosphatase, 4%. Of the labile P, 42% thus existed as ADP, corresponding to 1.1% of the labile P of the original ATP.

Allowing that the starting material had been subjected to some purification, and that the final product might still contain some ADP, the true ADP content cannot exceed this figure greatly, since larger amounts (up to 5% of the total labile P) would be discovered by direct enzymic assay of the main ATP preparation. By contrast, Kalekar (1947) infers that considerable amounts of ADP are present in guinea pig and hamster muscle.

DISCUSSION

The purity and stability of adenosinetriphosphate

The present results indicate that ATP preparations made by current modifications of the Lohmann method and precipitated 2-4 times are at least 95% pure. When freshly prepared they contain less than 1% inorganic pyrophosphate, which increases on storage at 0° at the rate of 1%/month. Both calcium and barium salts are subject to this change, and one sample of a dry sodium salt, stored at room temperature for a long period, had entirely decomposed. A less extensive breakdown to ADP and phosphate also occurs. If ATP samples have been prepared via the monocalcium or monobarium salt, the presence of free phosphate is itself indicative of extensive decomposition.

The complete enzymic assay of ATP preparations before use for other purposes may not always be possible. In this case, it is advisable to use ATP samples of known history and to check the extent of decomposition with yeast pyrophosphatase. This latter enzyme can be prepared in a form which is stable over many years, and the amounts required do not necessitate deproteinization.

It has not been possible to extend the investigation to the effect of water content on the decomposition of ATP salts, though negligible breakdown occurs when solutions of the sodium salt are stored at -10°. Recently, it has been found that ATP can be precipitated quantitatively as the benzidine salt

Table 4 *Enzymic analysis of the crude barium salts from trichloroacetic extracts of muscle*

Muscle (rabbit)	Mode of preparation	Phosphate split as percentage labile P	
		ATPase*	ATPase* myokinase
Fresh	Ba salts pptd at pH 6.5, washed twice	41	81
Fresh	As above, then pptd in ethanol HCl	45.5	91
Stood 5 hr at 15°	Ba salts pptd at pH 6.5, washed twice	35†	70†
Stood 5 hr at 15°	As above, then pptd in ethanol HCl	44	88

* ATPase = adenosinetriphosphatase

† Hydrolysis probably incomplete, owing to large amounts of inorganic phosphate present

concentration ever exists in muscle. Trichloroacetic extracts of fresh and rigor muscle were examined for the presence of ADP (a) by analysing the Ba salts precipitated at pH 7 and again after removal of free phosphate (b). No evidence of ADP in identifiable amounts was found in these crude fractions (Table 4). Method (a) suffers from the disadvantage that the large amounts of free phosphate present make

from aqueous ethanol at acid pH, and is quantitatively recovered by decomposing the complex with sulphuric acid, which forms the insoluble benzidine sulphate. The decomposition of barium-ATP, on the other hand, always involves some loss of nucleotide, whilst that of the acridine compound necessitates ether extraction. These advantages of benzidine as a precipitant for ATP will be enhanced if the complex is found to be more stable than the metallic salts of ATP.

The enzyme systems

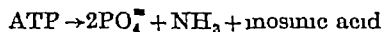
Whilst the use of myosin adenosinetriphosphatase alone, and in conjunction with myokinase, is in theory ideal for the determination of mixtures of ATP and ADP, the practical application is more difficult than was at first realized (cf. Bailey, 1948*a*). The main disadvantage lies in the slow hydrolysis of ATP by adenosinetriphosphatase in the later stages of the reaction, when the substrate is almost exhausted and inorganic phosphate has accumulated. Previous workers (e.g. Rapoport & Nelson, 1945) who have used myosin for the assay of ATP in biological material do not seem to have been aware of this effect, which is probably due to adsorption of ATP on the precipitated calcium phosphate. The long incubations necessary for the assay of ATP by adenosinetriphosphatase (7–10 hr at 25°) demand that the myosin be free from myokinase, and 4–5 precipitations are essential.

As an alternative to the use of myosin, potato apyrase is not entirely specific, and shows a feeble inorganic pyrophosphatase activity, and a still feeble action upon adenylic acid. Except for very precise analysis, these disadvantages are not serious. The usefulness of apyrase has been fully demonstrated by Kalckar (1944*b*), who has been able to differentiate the two labile phosphate groups of ATP in which ^{32}P had been incorporated. The terminal group was isolated by the yeast hexokinase reaction and both groups by the action of apyrase. The same differentiation or, if need be, the assay of mixtures of ADP and ATP, can be achieved by the use of hexokinase acting singly and in conjunction with myokinase (Kalckar, 1943). Unfortunately, the preparation of pure yeast hexokinase is a formidable task (cf. Bailey & Webb, 1948), and less pure preparations may be contaminated with adenosine triphosphatase (Kielly & Meyerhof, 1948). Kalckar (1947) later made use of apyrase in the development of an optical micromethod for the determination of adenine compounds, basing the method upon the marked change in the ultraviolet absorption when adenine, free or combined, is deaminated to hypoxanthine. In this way adenylic acid can be estimated after the action of muscle deaminase, ADP by myokinase in conjunction with deaminase, and ATP by apyrase and deaminase.

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Finally, some mention must be made of the claims of Banga (1947) and of Banga & Josepovits (1947*a, b*) with respect to the action of myosin on ATP. It is claimed that part of the ATP is degraded to ADP and part utilized in the formation of a dinucleoside pentaphosphate. The isolated dinucleotide shows some splitting in presence of myosin, but the production of inorganic phosphate is much greater in presence of myosin plus 'protein π '. The latter, as already pointed out (Bailey, 1948*b*), exhibits many of the properties of myokinase, and there is nothing in its manner of preparation which suggests its elimination. The obvious explanations for these results seem to have been meticulously avoided. First, in the action of myosin on ATP, only 33 % of the labile phosphorus is split, and the manner of isolation of the end products would give a product containing a mixture of ATP, ADP and inorganic pyrophosphate (if present). The renewed action of myosin itself on this mixture would affect only the ATP component, but acting in conjunction with protein π (myokinase), both ATP and ADP would be degraded to adenylic acid and inorganic phosphate.

Secondly, ammonia formation indicates the presence of adenylic acid deaminase in protein π . When the latter is added to the mixture, the ADP already present dismutates to ATP and adenylic acid by the action of myokinase, and the adenylic is at once deaminated. The deamination process itself speeds up the dismutation by removing one component of an equilibrium mixture, so that the initial formation of ammonia would be very rapid. This was actually observed. Later, ammonia production keeps pace with the splitting of phosphate, and this too can be explained, since the production of ADP, and hence of adenylic acid, is controlled by the hydrolysis of ATP by myosin, which becomes the limiting reaction. The overall reaction is



Expressed as a percentage of the total labile phosphorus and total amino nitrogen respectively, the liberation of free phosphorus and of ammonia must coincide. The results do not support the claim of Banga & Josepovits (1947*b*) that the action of myosin on ATP produces, in addition to the dinucleotide, an isomeric ADP in which a phosphate group is linked to the amino group of adenine. Not only are Banga's results unwarranted on the experimental evidence, but they can readily be explained in terms of known enzyme systems.

SUMMARY

1 The enzymic assay of adenosinetriphosphate and adenosinediphosphate, either singly or in mixtures, has been explored by use of myosin adenosine triphosphatase acting alone and in conjunction with

myokinase. A disadvantage of the method is the slow hydrolysis of adenosinetriphosphate in the final stages of the reaction.

2 Metallic salts of adenosinetriphosphate are shown to be unstable and break down on storage to adenylic acid and inorganic pyrophosphate, and to a less extent to adenosinediphosphate and inorganic phosphate.

3 The best preparations of adenosinetriphosphate are at least 95% pure. The possibility that some impurity is present is not excluded.

4 The use of potato apyrase for the assay of adenosinetriphosphate is restricted by its contamination with a nucleotidase and an inorganic pyrophosphatase.

5 Both in fresh and in rigor muscle, the adenosinediphosphate present is less than 5% and probably greater than 1% of the labile nucleotide phosphorus.

6 The claims of Banga concerning the action of muscle enzymes on adenosinetriphosphate are criticized.

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Preparation of the Antibiotic Nisin

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(Received 20 May 1940)

The early pharmacological work with nisin, prepared from *Streptococcus lactis* (Lancefield group N) (Mattick & Hirsch, 1947) was remarkable for the success which attended the use of crude concentrates. Nevertheless, it was obvious that more potent material would be required for human therapy and for experiments with the less sensitive pathogens. A note (Berridge, 1947) added to the paper of Mattick & Hirsch showed that highly active preparations in the form of crystals or tactoids could conveniently be made. The details of a number of such preparations are now described.

METHODS

(1) *The unit of antibiotic activity*. A preparation of nisin powder was chosen as a standard, and its activity defined as 1.3 units/ μ g; 1 unit will normally just inhibit the growth of

the test organism, *Strep agalactiae*, in 1 ml. of broth (test A₁), and is roughly equal to the unit of Mattick & Hirsch.

(2) *Measurement of antibiotic activity*. Three tests were given a prolonged trial during this work. A, Bacteriostatic tests: (i) dilutions of the antibiotic in powers of 2 in inoculated broth, with the recording of growth after 16 hr and 40 hr as +, ++ or +++; (ii) dilution of the antibiotic in powers of 1.3–1.5 in broth or skim milk heavily seeded with sensitive acid-forming organisms with the recording of pH after 10–16 hr incubation, and B, an apparently bactericidal test in which the proportion of organisms surviving a few minutes' contact with the antibiotic diluted in powers of 4 was determined by the plate count. It is quite possible that different activities were measured by the different methods. The details of the methods are as follows.

Method A₁. This did not differ significantly from that described by Mattick & Hirsch (1947).

Method A₁₁. The main samples were diluted on the basis of an assumed activity to give solutions with about 10 units/ml. Suitably spaced volumes (e.g. from 0.08 ml. increasing

by powers of 1.5 to 1.20 ml) were measured from a micro-burette into a series of sterile test tubes. For each set of determinations carried out on the same day with the same preparation of test organism, a standard nisin solution freshly diluted to 10 units/ml was dispensed similarly but over a smaller range and at closer intervals (e.g. from 0.1 ml rising by increments of 0.03 ml to 0.70 ml.) Pasteurized skim milk tinted with litmus was inoculated with 1% (v/v) of a 24 (± 3) hr culture of *Strep cremoris* (strain 1P5) and 10 ml. mixed with the nisin solutions. The tubes were then incubated at 22°. The progressive reduction of the litmus by the growing micro organisms, and the final clotting of the milk, enabled the state of each test to be observed at a glance. After about 10 hr incubation pH determinations were made on each partly grown culture. From the graph of nisin concentration against pH, drawn from the standard, it was then possible to determine the activity of the unknown solutions. If incubation was too prolonged the graph from the standard test became too steep, there being then only one or two intermediate points between which to draw the useful part of the curve. The same technique has been followed using yeast dextrose broth in place of milk. The heavy inoculum, and the rapid growth of the test organism at a comparatively low temperature, made it possible to dispense with most of the usual precautions of asepsis, but samples were freed from gross contamination by being diluted in 0.05N HCl.

The uncertainties in constructing and using the standard graphs gave an error of less than $\pm 10\%$ in the final figures. The method was based on a suggestion of Hirsch, which he has since subjected to considerable study and elaborated into a 'lag phase assay' (using *Strep agalactiae*), the reproducibility of which has been statistically analysed (Hirsch, 1949).

Method B. A 0.001% suspension of a 24 (± 3) hr culture of *Strep agalactiae*, giving a plate count of 3000–6000/ml, was allowed to stand for 6 min in contact with serial dilutions of standard nisin diminishing from 50 units/ml in powers of 2, and of unknown solutions diminishing from about 100 units/ml in powers of 4. 0.1 ml of each mixture was then plated with 10 ml of melted cooled agar and counts made after 24–48 hr incubation at 37°. Reductions of 30–70% in the count usually occurred between 6 and 50 units/ml.

A rough graph was made by joining with straight lines the points obtained when x was plotted against y , where $x = \log(100a/b)$, a = number of organisms surviving contact with nisin, b = number of living organisms before adding nisin, and y = nisin concentration in contact with the organisms in the broth suspensions. The activities in the unknown solution were then obtained by interpolation. Although the variations from day to day were great, and although there were factors of unknown magnitude such as the number of organisms killed in, for example, tube no. 2 before transferring 1 ml to tube no. 3, it was anticipated that they would be overcome by the preparing of a standard graph for each set of determinations. However, this was not so, for after a few months trial the method appeared only a little better than method A₁ and was therefore abandoned in favour of A₁ for ordinary, and A₂ for special purposes, although by this time it had been used for most of the experiments in purifying and crystallizing nisin. The plate counts of *Strep agalactiae* have shown a reproducibility unusual with long-chained organisms. This and other aspects of the 'bactericidal action' of nisin have been examined in detail by Hirsch (1949).

At an early stage of the work the use of *Strep cremoris* in a test like A₁ led to the preparation of a concentrate active against this organism but without effect upon *Strep agalactiae*. Therefore test A₂ with *Strep cremoris* has been used only when its different sensitivity was not likely to be misleading.

(3) *Determination of specific activity* Samples, usually of 0.1 ml, of nisin solutions were dried at 98–100° in small platinum dishes, weighed on the microbalance, ashed, and reweighed as described elsewhere (Berridge, 1945). The specific activities were calculated in units/ μ g of organic matter.

(4) *Determination of nitrogen* The micro Kjeldahl method of Tompkins & Kirk (1942) was used but with the digestion mixture of Campbell & Hanna (1937).

(5) *Apparatus for cooling at a controlled rate* This was used for some of the later crystallizations.

A contact thermometer (electro methods) in which the temperature setting could be controlled by rotating a magnet, was set up in a thermostatically controlled water bath. A train of Meccano gears and chain drives was then arranged between a synchronous electric motor and the controlling magnet so that the temperature of the water bath could be changed at any desired rate. The small fluctuation permitted by the contact thermometer appeared to be unimportant. With the help of a cooling coil connected to the main water supply, temperatures somewhat below room temperature could be reached.

(6) *Diffusion measurements* Diffusion was allowed to take place through a sintered glass disk according to the technique described by Northrop & Anson (1929) (see also Gordon, 1945). Special precautions were taken to overcome vibration, and the temperature was regulated at $25 \pm 0.003^\circ$. The vessel was standardized with 0.1N KCl using the value for the integral diffusion coefficient $D_i = 183.8 \times 10^{-7}$ cm²/sec (Gordon, 1945). Measurements with nisin were made by comparing the activities of the inner and outer solutions according to method A₂.

(7) *Solubility determinations* The nisin suspensions were allowed to reach equilibrium in bottle shaped vessels of about 0.5 ml capacity, each containing a newly made glass bead of smooth bright surface, which stirred the suspension while the bottle was rotated in a vertical plane. The suspensions were transferred to the bottles with a Pasteur pipette having a waxed tip. By filling exactly to the neck it was possible to avoid bubbles and at the same time to achieve adequate stirring. In order to avoid a partial vacuum in the bottles after sealing, the necks were first pulled out to very fine tips, allowed to cool and finally sealed with a minute flame. The sealed bottles were packed in cotton wool and immersed in crushed ice in a quart Thermos flask which was rotated in a vertical plane. After equilibrium had been reached the suspensions were allowed to settle for several days and the supernatant solutions removed by pipette. This process was repeated to ensure freedom from contamination by traces of solid, and the nitrogen content of the solutions determined.

PREPARATIONS

Preparation of crude concentrates The nisin producing organism was a *Strep lactis* (Lancefield group N) strain M354/07, identical with that used by Mattick & Hirsch (1947). It was propagated by daily subculturing in yeast dextrose lemco broth, in which medium nisin was also

readily formed. Stock cultures were kept in bullock's heart medium in the refrigerator.

Concentrates were made by a method similar to that of Mattick & Hirsch, viz adsorption on to a CHCl_3 emulsion followed by removal of the CHCl_3 . After growth the broth was acidified to $\text{pH } 1.9 \pm 0.1$, allowed to stand for 1 or 2 days and siphoned off. The pH was raised to 5.0 and 3% (v/v) CHCl_3 emulsified with the broth and kept suspended for about 30 min. After 2 hr settling the supernatant liquid was siphoned off. The residue was acidified with 0.12 ml of 10N HCl /l of original broth, the CHCl_3 distilled off *in vacuo* below 40° and the remaining suspension filtered at $\text{pH } 1.5 \pm 0.1$. The filtrate was boiled to destroy possible enzymes and concentrated fivefold *in vacuo*. An inactive precipitate was removed by raising the pH to 1.9 ± 0.1 and adding 2 vol of ethanol and 1 of ether, the mix itself was precipitated with a further 3 vol of ethanol and 5 of ether and dried with ethanol and ether. Powders so prepared usually had specific activities of about 1 unit/ μg .

Fractionation in phosphate buffer It was found that a product of seven or eight times the specific activity of the crude material could be prepared by treatment with M K phosphate at pH 6.1, most of the activity remaining in the small insoluble portion. A solution of precipitate prepared in this way had a specific activity of 8 units/ μg , and a dry powder with a specific activity of 8 units/ μg was obtained by precipitation with alcohol and ether.

The effect of pH on the fractionation was explored in an experiment in which 0.56 g of crude powder (kindly supplied by Bengers Ltd) was suspended in 12 ml M H_2PO_4 , and precipitates were obtained and separated serially as the pH was raised from 1.4 through 2.3 and 3.8 to >6 and <7 . After dissolving the precipitates in 0.05N HCl their activities and organic dry weights were determined, giving the results shown in Table 1, from which it is clear that fractionation at the lower pH values gives less increase in purity than was obtained in the previous experiments.

Table 1 *Fractionation in molar potassium phosphates at various pH values*

	Total activity (units $\times 10^6$)	Specific activity (units/ μg)
Original powder	0.6	1
Fraction		
(1) Precipitate at pH 1.4	0.6	3.4
(2) Precipitating between pH 1.4 and 2.3	0.2	3.8
(3) Precipitating between pH 2.3 and 3.8	0.1	1.9
(4) Remaining soluble at pH 3.8 to >6 and <7	0.08	0.2
Recovered in fractions	0.98	

The increase in total activity shown in Table 1 contradicted the first four experiments in which the yields appeared to be very low. The gains occurring in the following detailed example of the fractionation at pH 6.1 in M K phosphate were therefore determined as accurately as the method of activity measurement (b) would allow. Nissn powder (5.35 g, 0.9 unit/ μg) was dissolved in 106 ml of 0.1N HCl giving a turbid mixture of pH 0.9. The precipitate obtained on centrifuging dissolved readily in 0.05N HCl and was added to the nissn residues. To the supernatant liquid

sufficient KH_2PO_4 was added to bring the concentration to molar, i.e. 14 g, and after solution, the pH was raised to 6.11 with concentrated KOH (about 10 ml). The precipitate, which began to form before the pH reached 3, increased until about pH 5, and decreased again greatly by pH 6. That which remained was allowed to stand until well flocculated, but even so the supernatant liquid obtained by centrifuging was not clear. A clear supernatant was produced, however, by a second centrifuging after the liquid had been allowed to stand overnight in the refrigerator. Thus a main and a subsidiary precipitate were obtained. The main precipitate was dissolved in 30 ml of distilled water and adjusted with HCl to a final pH of 1.9. The solution had an activity of 250,000 units/ml. The total activities existing at the various stages are recorded in Table 2.

Table 2 *Increases in activity during fractionation in phosphate buffer*

	Total activity (units $\times 10^6$)
Starting material (5.35 g at 0.9 unit/ μg)	— 4.8
Solution in 0.1N HCl (A)	4.6
Precipitate from 0.1N HCl	1.6
Solution (A) after adding phosphate and adjusting to pH 6.1 (sample 'activated' by standing overnight in 100 ml 0.05N HCl)	— 5.5
Solution of main precipitate	7.8
Solution of subsidiary precipitate	0.4
Supernatant buffer solution (after acid 'activation' as above)	0.3
	8.5

In this experiment, therefore, 7.8×10^6 units, with a specific activity of presumably about 8 units/ μg , were prepared from 4.8×10^6 units, with a specific activity of 0.9 unit/ μg . This apparent increase has been confirmed but not further investigated.

Further purification by fractional precipitation with sodium chloride An exploratory fractionation of the solution from the above experiment was made by dissolving in it gradually increasing quantities of NaCl , centrifuging as soon as a fifth to a tenth of the nissn appeared to be precipitated, and repeating the process until no further precipitate appeared. In this way five roughly equal fractions were obtained. Their specific activities were respectively 20, 18, 27, 24 and 5.0 units/ μg . There seemed to be a loss of about 50%, but the activities of some of the acid solutions of the precipitates increased on keeping. Attempts to purify these fractions further by other methods, which are described below, were unsuccessful. Purification was therefore again attempted with fresh material.

Crude nissn powder was fractionated with phosphate buffer as already described, giving a solution of purified nissn in dilute HCl at pH 2. A slight precipitate produced by adding NaCl was discarded, and the bulk of the nissn then precipitated by saturation with NaCl and redissolved at pH 1.8. From 10 million units of crude nissn 130 ml of purified solution containing 17 million units were obtained, an increase as before. Seven portions of NaCl were added in turn to this solution, the precipitate salted out by each being collected for the determination of total and specific activity. The procedure and results are summarized in Table 3. The final solution at pH 1.0 contained an insignificant quantity of nissn.

Table 3 *Fractionation of nisin solutions by precipitation with sodium chloride*

(Original solution 17×10^6 units in 130 ml. at pH 1.8, prepared by phosphate fractionation and preliminary precipitation with NaCl. NaCl (x g) was added to supernatant number (Y) to give precipitate number (Y + 1))

Supernatant number (Y)	NaCl added (x g)	Precipitate number (Y + 1)	Resuspended precipitate (Y + 1)		
			Specific activity (units/ μ g)	Total activity (units $\times 10^6$)	pH
0	4.5	1	20	—	2.3
1	1	2	30	2.9	—
2	1	3	23	1.7	2.5
3	1.5	4	31	1.4	—
4	3	5	34	2.6	2.6
5	4.5	6	17	1.0	—
6	15	7	16	0.9	2.8
7	—	—	—	—	1.0

Thus, considerable increases in specific activity could be achieved by fractionation with NaCl.

Crystallization Precipitates 2, 3 and 4 (selected before the specific activities had been determined) were united (20 ml in all), adjusted to pH 2 and the solution saturated with NaCl. The resulting precipitate was washed with 3 ml. of distilled water, and then extracted three times with distilled water containing sufficient NaOH to give, in the mixture, a pH of 6.2, as described for extractions at pH 5.6 under 'Other methods of concentration and fractionation' (p. 491). Numerous attempts were made to crystallize these extracts by various techniques, especially by several different ways of slowly adding NaCl. The precipitates obtained were always granular and amorphous, but occasionally they contained a minute proportion of straight needles. The third of the extracts had been precipitated with NaCl several times at pH 2 when crystallization from ethanol was attempted. An equal volume of absolute ethanol was added to the centrifuged precipitate causing its immediate solution, followed by a slower precipitation which was reversible and dependent on temperature. On cooling the solution gradually from 37 to 0° a semi-solid suspension of long thin needles was formed. This was filtered in the refrigerator through a small disk of filter paper (about 1 cm. diam.), washed with a little ice-cold 80% (v/v) ethanol, and dissolved in 1 ml. of 0.05N HCl. Triplicate determinations of the organic dry weight of this solution gave 0.65, 0.61 and 0.63 mg./0.1 ml. The activity was 4000 units/0.1 ml. Thus the specific activity of the crystals or tactoids was 73 units/ μ g.

Further preparations of crystalline nisin Comparatively large quantities of 'crystalline' nisin were needed for animal experiments, and a series of eight preparations were next made from dry powders at 10 units/ μ g., which were now being produced by a small pilot plant.

The preparations were made according to modifications of the method described under 'Final preparation' (p. 490). In the first four of them particular attention was paid to the effect of various modifications on the yield. These yields, based not on weight but on activities, were respectively 24, 15, 26 and 15% of the starting material, but to avoid prolixity the experience of the whole series of preparations is summarized in the following points.

(1) It was thought that previous failures to crystallize from aqueous media might have been due to impurities which ethanol crystallization would remove, but the 'crystalline' substance transferred to 0.05N HCl and salted out with 0.25 saturated $MgSO_4$ solution reappeared in

the amorphous condition, although the $MgSO_4$ was added so slowly that 6 days were required for the precipitation.

(2) Most of the preparations contained a small quantity of gummy substance which would not dissolve in 80% ethanol but which could be removed by filtration at 37°, a process requiring about 24 hr. An attempt to increase the yield by increasing the middle fraction at the expense of both of the others led to such an increase in the quantity of the gummy substance that filtration was practically impossible. However, repeated extraction with 80% ethanol at 37° by centrifuging gave clear liquids which 'crystallized' well, but the yield was low.

(3) The separation of too little of the least soluble fraction led to a middle fraction which remained amorphous in definitely during repeated 'recrystallizations'.

(4) Considerable quantities of nisin could be recovered from abortive experiments, and from mother liquors and other ethanolic residues by diluting with 5 vol. of distilled water, precipitating by saturation with NaCl and fractionating according to the simple unmodified scheme.

(5) Unless the properties of the starting material rendered the least soluble fraction inordinately large its further fractionation was uneconomic. 'Working up' processes of varying complexity, but always on the lines of the main preparation, were tried out on the least soluble fractions of three preparations. From only one was a significant increase in yield obtained.

(6) Nisin can be precipitated from dilute solution in 80% ethanol by raising the apparent pH to about 6, but whenever this was done there were great losses in activity.

(7) The correct acidity is essential for the formation of regular needles. Different quantities of HCl and NH_3 were added to samples of a suspension which had precipitated in the usual form of very long thin needles. The less acid suspensions needed considerable dilution before they could be brought into solution. After warming to dissolve and cooling slowly the precipitates were in the form of irregular thin crumpled plates from solutions of pH 2.42, 2.74 and 3.00, and microscopic needles at pH 4.22 diminishing again to microscopic threads at pH 4.74.

These observations suggest that the losses sometimes experienced as a result of difficulty in redissolving the needles in 80% (v/v) ethanol could have been avoided by using acidified ethanol and adjusting the pH after solution.

(8) The size of the needles could be considerably increased at the optimum acidity by controlling the rate of cooling. All arrangements of lagged vessels involve, at the

beginning of the process, a comparatively rapid cooling which leads to the formation of numerous small particles. With the apparatus already described (see Methods) this effect may be avoided without unduly prolonging the period of cooling. When cooled at the rate of $0.5^{\circ}/\text{hr}$ a sample which had previously given 'crystals' appearing under the microscope ($\frac{1}{2}$ in objective) as fine needles now formed a precipitate recognizable as needles with some difficulty by the naked eye, but with ease at a magnification of 10 diameters.

(9) Several other methods of crystallization were tried without success, such as, for example, allowing an aqueous solution to evaporate in a desiccator at atmospheric pressure, removing water from a solution in 80% (v/v) ethanol by keeping in a desiccator over Ca metal (nisin is soluble in 80% but not in absolute ethanol), and stirring an acid ethanolic solution in a desiccator over dilute ammonia.

Final preparation Nisin powder, 17.4 g at 10 units/ μg , was suspended in 700 ml of distilled water by gentle stirring during about 30 min. The addition of 3 ml of concentrated HCl (36% w/v) brought all but traces of the nisin into solution to produce a brown turbid liquid. After its pH had been brought up to 1.8 with a little 15% (w/v) ammonia, 28 g NaCl were dissolved in the nisin solution and the

resulting dark brown gelatinous precipitate filtered off immediately with Whatman no 54 paper. Notwithstanding the increasing opalescence of the filtrate resulting from equilibrium not having been established, a further 4.4 g NaCl/100 ml, actually 29 g in 650 ml, were added. The precipitate which now formed was stirred in its mother liquor for about 30 min before being filtered off (again no 54 paper, both these filtrations were very rapid). The cream coloured semi solid precipitate was easily removed from the paper and was resuspended in enough distilled water to give a volume of 360 ml., in which it dissolved slowly. One half volume of saturated NaCl solution was now added slowly with continual stirring. By repeated gentle centrifuging the resulting precipitate was easily consolidated in one tube where it was dissolved by warming and shaking with about 1.2 vol of absolute ethanol to give 150 ml. of clear brown solution. The 'pH' ('pH' = Glass electrode reading of 0.1 ml ethanolic solution + 1.1 ml. distilled water) of this solution was raised with dilute ammonia from 3.5 to 4.21, a procedure which diminished the solubility of the nisin so that it was now necessary to warm it to 45° before complete solution occurred. On cooling from this temperature at the rate of $1^{\circ}/\text{hr}$ without stirring, comparatively large needles were formed. The brown colour remained in the mother

Table 4 *Purification of nisin*

Procedure	Quantity (units $\times 10^7$)	Purity (units/ μg)
17.4 g powder in 700 ml + 28 g NaCl	21	12
Precipitate (discarded)		
Filtrate	17	18
+ 29 g NaCl		
Filtrate (discarded)		
Precipitate	12	24
Dissolved in water to 360 ml + 180 ml saturated NaCl solution Centrifuged		
Supernatant (discarded)		
Precipitate	14	26
Dissolved in ethanol to 180 ml at 45° and 'pH' 4.2 Cooled at $1^{\circ}/\text{hr}$ Centrifuged		
Mother liquor (to residues)		
Needles	11	40
Redissolved in 80% (v/v) ethanol at 37° and 'pH' 4.2 Filtered at 37° and recrystallized by slow cooling		

liquor On resuspending the precipitate in 80% (w/v) ethanol a mixture of 'pH' 4.7 was obtained and some difficulty was encountered in achieving complete solution even at 'pH' 4.2 Although 80% (w/v) ethanol was added until the volume of suspension was 280-300 ml some insoluble particles indistinguishable from the needles remained (Probably better results would have been obtained by dissolving the precipitate at 'pH' 3.0-3.5 in a volume equal to that of the mother liquor and raising the 'pH' to 4.2) The dilute solution was filtered overnight at 37° before sampling for yield and purity On cooling at 0.5°/hr it gave needles similar to the former ones but practically white in colour They were smaller than those mentioned in (8) above The process is summarized in Table 4 At this early stage it cannot be stated whether the discrepancy between the specific activity of this material and that of the 'crystals' first obtained arose from errors in the measurements of activity, from errors in standardization, or from actual differences in composition

Other methods of concentration and fractionation At pH values near neutrality crude nisin was adsorbed from the broth in which it had been produced on to each of all the insoluble substances tested, e.g. aluminium hydroxide, charcoal of all grades, benzoic acid (pH 3-4), emulsified ether, ethyl acetate, CHCl_3 , CCl_4 , trichloroethylene and cellulose (filter paper pulp) It was always difficult to recover the nisin from the adsorption complex Occasionally, as with cellulose, it could be obtained by extraction at pH 1.8, but even so the losses were high.

From solution at about 1000 units/ml nisin was readily precipitated by a variety of protein precipitating reagents such as tannic acid, phosphotungstic acid and uranyl acetate, but again the difficulty was the recovery of the material in an active condition

Further fractionation of the concentrated, partly purified material (200,000 units/ml, 8 units/ μg) by partial precipitation of an ethanolic solution with ether gave some promise of success, but was eventually abandoned as unattractive

Repeated extraction of precipitated nisin at pH 5.6 with distilled water gives results similar, but perhaps inferior, to those from partial precipitation with NaCl In a preliminary experiment beginning with a preparation at 8 units/ μg it was possible to account for roughly 50% of the activity, while the last extract had a specific activity of 27 units/ μg A second experiment was made in which 4.4 g of powder at 1 unit/ μg were subjected to fractionation in phosphate buffer (see p. 488), the nisin being precipitated from the final solution at pH 1.7 by saturation with NaCl The precipitate was extracted by 'creaming' it with distilled water, diluting to about 20 ml with distilled water, adjusting its pH to 5.5-5.7, and centrifuging The procedure was repeated twice, using the remaining residue each time Three extracts and one residue were thus obtained The first extract only was further purified by centrifuging away the first turbidity which appeared when NaCl was added very gradually precipitating the remaining nisin by saturation with NaCl and extracting this precipitate once with distilled water at pH 5.5 All solutions were now precipitated by saturating with NaCl and centrifuged These and the other precipitates were finally dissolved in 2.5 ml 0.05N HCl before testing their activities The results are shown in Table 5 Neither these nor those of Table 3 are adequate for a comparison of the methods because the differences are not great In the absence of further evidence fractionation

with NaCl is, however, to be preferred on account of the greater stability of nisin at the lower pH values there employed

Table 5 *Fractionation of nisin at pH 5.6*

	Quantity (units $\times 10^3$)	Specific activity (units/ μg)
Original powder	4.4	1.0
Fractions		
Phosphate fraction	—	8*
First extract		
First turbidity with NaCl	0.07	—
Extract at pH 5.5	0.22	7.7
Precipitate at pH 5.5	0.26	—
Second extract	1.4	23
Third extract	0.79	23
Residue	0.71	—
Total recovery	3.45	

* Assumed on the basis of other experiments

PROPERTIES OF NISIN

The biological properties of crude nisin preparations have already been described (Mattick & Hirsch, 1947; Hirsch, 1949)

Stability Dry nisin powder appears to keep indefinitely in an ordinary atmosphere At pH 6.8 and 37° in dextrose leuco broth nisin retains its activity for at least 24 hr, solutions at pH 1.8 are stable for weeks Boiling at pH 1.8-2.0 for 5-10 min tends, if anything, to increase slightly the activity of dilute nisin solutions Moreover, concentrated solutions prepared by dissolving 'crystals' from ethanolic solution in dilute HCl and adjusting to pH 2.0 may be boiled in an open beaker until free from ethanol without appreciable loss of activity, and saturated solutions at pH 4.2 may be 'sterilized' for injection by boiling for 10 min Autoclaving, however (10 lb/sq.in for 10 min), destroys about 50% of the activity In boiling 2N HCl destruction is complete after 2 min In alkaline solutions the activity is quickly destroyed An acid solution was immediately inactivated by potassium permanganate but not by sodium sulphide

Nisin was suspected to be a peptone It appeared possible therefore that it would be rapidly destroyed in an enzyme rich medium such as blood Preliminary experiments showed that nisin could be assayed in blood using method A₁ with an inoculum of 1% of a 24 hr culture of *Strep. agalactiae* Method B could also be used It was then demonstrated by the former method that 6000 units/ml in blood at 37° remained undiminished for at least 22 hr Method B was then used to demonstrate an equal stability of nisin under similar conditions at a level of 15 units/ml Fresh citrated whole blood was used

Solubility (1) *In blood* As nisin was known to have a comparatively low solubility at pH levels near neutrality its solubility in blood became of interest as a factor which might limit its therapeutic use After the preliminary tests described above method B was used to measure, first, the distribution between corpuscles and plasma and, secondly, the activity of plasma after adding various quantities of nisin to blood and centrifuging It was found that the nisin was distributed evenly between the plasma and the corpuscles, medium concentrations remaining unaffected by

centrifuging, and that the 'solubility' in blood is not less than 5000 units/ml, a value not too low to prevent its therapeutic use

(ii) *In water* At low pH values nisin is very soluble. At a pH about 4.2 saturated solutions of 'crystalline' nisin had an activity of 600,000 units/ml, corresponding to a solubility of approximately 1.5% (w/v). In extraction experiments nisin at about 20 units/ μ g gave solutions containing 40,000 units/ml at pH 5.6, but here adsorption effects may have interfered.

(iii) *In organic solvents* Preliminary qualitative tests indicated that those compounds which dissolved nisin usually destroyed its activity, e.g. glacial acetic acid, benzyl alcohol. Ethanol (80% w/v) and formamide were exceptions, in that full activity was retained.

Molecular size A rough measurement of the diffusion coefficient of nisin was made using the sintered disk method. This method was chosen because the results can be obtained from activity determinations alone, inactive impurities causing only indirect effects, such as, for example, the possible effect of rapidly diffusing molecules on the viscosity of the liquid into which a slower molecule might diffuse. It was, furthermore, impossible to find laboratory accommodation free from vibration. The details of the arrangement employed are described under Methods (p. 487).

A preliminary single determination of the diffusion coefficient gave 15×10^{-7} cm²/sec. In the next experiment diffusion was allowed to proceed for a total of 45.7 hr, the outer liquid being renewed at intervals. It is probable that the differences which still appeared (see Table 6) after equilibrium should have been established were due to uncertainties in the biological assay. Thus the value of 14×10^{-7} cm²/sec for the diffusion coefficient of nisin is merely approximate. Nevertheless, it serves to show that nisin is probably a small protein or large polypeptide molecule (cf. *D* (diffusion coefficient at infinite dilution) = 10.57×10^{-7} for lactalbumin, 10.11×10^{-7} for cytochrome c Polson, 1939, Schmidt, 1943).

precipitated from ethanolic solution, was dissolved in 0.05N HCl and salted out by the slow addition of NaCl through a rotating membrane (McMeekan, 1939). The resulting granular precipitates were convenient for settling, filtering and washing. The material for the first experiment was equilibrated with buffer by five washings on a sintered glass filter, that for the second by dialysis with renewal of buffer daily for 13 days. After this second procedure, 25% of the nisin was discovered to be insoluble, probably chemicals from the insufficiently washed collodion membrane brought

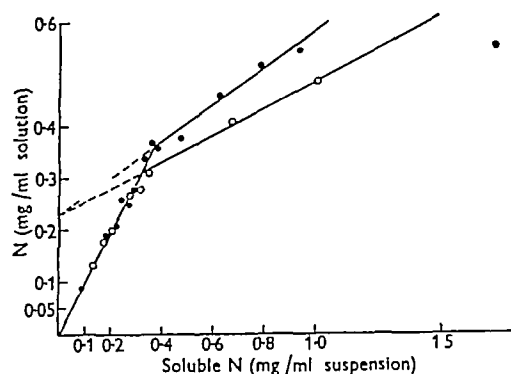


Fig. 1. Solubility of nisin: ●, Exp. 1; ○, Exp. 2.

about denaturation, 25% was therefore subtracted from the figures for total N in the second solubility experiment. The buffer was made by dissolving 26 g. KH₂PO₄ and 60 g. KCl in 800 ml. of distilled water, adding 10N HCl to pH 1.92 and finally making up to 1 l. Nisin suspensions of different concentrations were made, and after equilibrium had been established the solutions were separated and analysed for nitrogen as already described (see p. 487). Both sets of results are shown graphically in Fig. 1, from which it is clear that these preparations contained at least two components,

Table 6. Diffusion of nisin

(Inner solution, nisin, 65 ml at 175,000 units/ml. Outer solution, solvent, 25–50 ml.)

Diffusion no	Time during which diffusion occurred (hr)	Units diffused	Units diffused/hr	Diffusion coefficient (cm ² /sec)
1	4.5	81,000	18,000	— 14 × 10 ⁻⁷
2	17.1	94,000	5,500	
3	12.0	80,000	6,700	
4	12.1	85,000	7,000	
			Mean 6,400	

Amino acid composition Paper partition chromatography (Consden, Gordon & Martin, 1944) was used to analyse a hydrolysate of 'crystalline' nisin. The following amino acids were recognized, alanine, valine, leucine, iso-leucine. Cystine and aspartic acid were possibly present. There were also several faint or unrecognizable spots.

Purity The solubility of nisin depends on the quantity of solid phase present, when the concentration of the solution was plotted against that of the suspension two straight lines of different slope were obtained showing that the preparation contained at least two components. A solution saturated with respect to all components was not obtained.

Two main experiments were carried out. For each of them a different preparation of nisin, in the form of needles pre-

the less soluble of which amounted to about two thirds of the total mixture. The possibility mentioned by Butler (1941) that different forms of one component may persist although not in equilibrium, remains to be examined, but it seems remote because of the slow precipitation of the solid used in these experiments.

DISCUSSION

Throughout the work there were a number of unexplained observations which could not then be further investigated. The increases in activity recorded in Tables 1 and 2 are among them. Some

of the others may be merely mentioned, for example (a) the activity of acidified broth containing nisin was destroyed on attempting to concentrate *in vacuo*, some activity reappearing on keeping, (b) when the organism was allowed to grow at 22° instead of 30° a concentrated product active against *Strep cremoris* but not against *Strep agalactiae* was obtained, (c) significant yields were obtained in some media after steaming, but not after autoclaving, although the organisms still grew well, and (d) the sensitivity of nisin to certain chemicals and solvents contrasts strangely with its stability to others and to acids and heat

It was impossible to prove that the needles observed were true crystals. Their shape in cross section could not be observed. The slow filtering and the slightly syrupy nature of the ethanolic solutions, as well as the formation of gels when such solutions were rapidly cooled, suggests that the needles may well have been tactoids.

Although a few preliminary experiments have shown that nisin is closely associated with a polypeptide, the solubility measurements indicate that further purification is required before the properties of the active substance, or substances, can be properly investigated. Meanwhile, the purification so far achieved has facilitated experiments in other directions.

SUMMARY

1 Experiments in concentrating and purifying the antibiotic nisin are described

2 Nisin may be obtained in the form of needles by cooling a solution in 80 % ethanol at 'pH' 4.2 ('pH' is glass electrode reading of ethanolic solution diluted with 11 volumes distilled water)

3 The properties of the 'crystalline' product have been investigated. It will inhibit the growth of the more sensitive organisms at dilutions from 10^{-8} (*Strep agalactiae*) to 10^{-9} (*Strep cremoris*), it is stable for weeks in acid solution and for many hours in blood at 37°, it contains the amino acids alanine, valine, leucine, isoleucine and others, and appears to consist of a mixture of at least two polypeptides.

Special thanks are due to Benger's Ltd. (of British Chemicals and Biologicals Ltd.) for their co-operation in making, and generosity in supplying, active concentrates. The helpful encouragement given by the Director, Prof. H. D. Kay, and the invaluable support of the Assistant-Director, Dr A. T. R. Mattick, are gratefully acknowledged. Thanks are also expressed to Dr A. Hirsch for useful suggestions. The work described under 'Preparation of crude concentrates' was carried out in the laboratories of Benger's Ltd., whose Director, Mr B. D. Thornley, is to be thanked, as are also Dr E. G. Stopher, Mr C. Epstein, and Miss E. M. Barnes, members of the staff, for their assistance. The later help of Mrs C. Waterhouse is acknowledged with thanks.

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The Cholinesterase Activity of the Serum of Newborn Animals, and of Colostrum

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(Received 23 May 1949)

The activities of the enzymes in serum which split acetylcholine have been investigated fairly extensively in man, both in health and disease. Enough work has also been carried out on animal sera to establish the general level of enzyme activities in the various species, and active preparations of the so-called 'pseudo' enzyme (Mendel, Mundell

& Rudney, 1943) have been made from various tissues. It is, however, believed that no tests have hitherto been made on newborn animals, or on the colostrum and milk of their mothers. Human beings, rats, rabbits, guinea pigs, cats and dogs have been investigated in the work to be described.

METHODS

The adult humans were healthy males. Blood was taken from them by vein puncture with a minimum of stasis. The infants were of both sexes and were all full term and normal. Their mothers were also normal and had given birth to the children without assistance or anaesthetic. Blood was taken from infants at birth by puncturing the umbilical cord with a large bore needle or by allowing the maternal end of the cord to drain into a centrifuge tube, and later from the heel by cutting out a disk of skin with a scleral trophine and allowing the blood to flow without pressure into a small tube. Blood was obtained without anaesthesia from adult animals by vein puncture or decapitation, and from young animals by heart puncture from the following species: rats (Lister Institute strain), guinea pigs and rabbits (Department of Pathology stock), cats and dogs of various breeds. At birth and during the first 7 days thereafter, blood had to be withdrawn from several rats to get enough serum for each determination. After the samples were taken, the serum was separated by centrifuging as soon as possible, with care to minimize evaporation. Puncturing the heart did not appear to harm the young animals, and, after a few days, second and even third samples of blood were sometimes taken from the same animal by this method. Colostrum and milk were obtained by manual expression.

The measurements of enzyme activity were made manometrically at 37° (McArdle, 1940), using 1 ml of 2.5% acetylcholine chloride as substrate in a total volume of 3 ml of fluid. The liberation of 1 μ l of CO₂/min/ml serum, colostrum or milk has been defined as the unit activity.

RESULTS

Man. The average activity of 28 adult sera was 76 units (s.d. 13.8), which agreed very well with the figure obtained by McArdle (1940). The mean activity of 39 cord sera was 50 units (s.d. 10.7). This difference was significant ($t=12.50$, $P<0.01$). The changes in activity following birth are shown in Table 1.

Table 1 *The cholinesterase activity of the sera of newborn infants*

Age	No of samples	Average activity (μ l CO ₂ /ml/min)	Standard deviation
At birth	39	50	10.7
0-24 hr	39	62	14.1
5-10 days	71	64	15.7
11-21 days	19	75	14.3
Over 3 weeks	4	87	2.7

The apparent rise in the first 24 hr was checked on numerous occasions by taking both specimens of blood from the same baby. The increase in the average activity of the sera of the 39 babies from 50 to 62 units was statistically valid ($t=6.93$, $P<0.01$). This rise, however, may not represent a real change in enzyme activity, since the two specimens of blood were not taken from the same site, and peripheral blood has been shown to be more

concentrated, or at any rate to carry more haemoglobin/ml, than cord or sinus blood (DeMarsh, Alt, Windle & Hillis, 1941; DeMarsh, Alt & Windle, 1948). These observations have been confirmed. The subsequent rise of enzyme activity was to be expected at some period of early childhood, since McArdle (1940) found older children to have higher serum activities than adults and this has been confirmed by one of the authors (Hutchinson, 1949). No

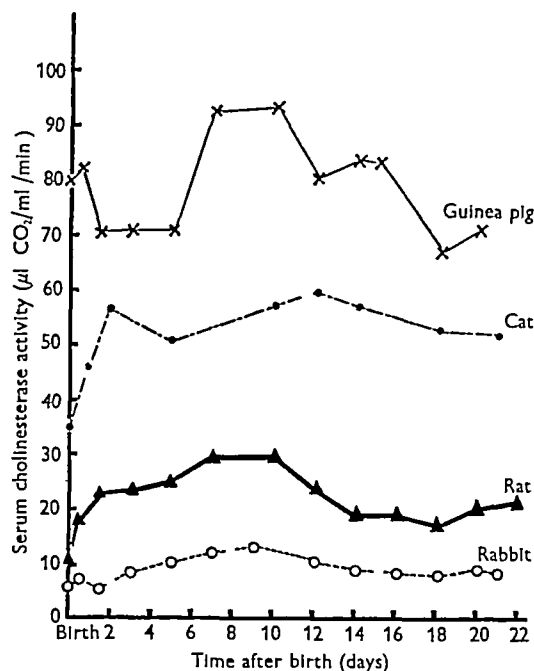


Fig. 1 Cholinesterase activities in the sera of young animals

relationship has been found between the level of activity in cord sera and the weight of the child at birth, or the length of its gestation period as reckoned from the beginning of the mother's last menstrual period. No correlation has been found, moreover, between the enzyme activity of cord sera and the mother's age or her clinical status. The level of enzyme activity of colostrum, and milk up to the 22nd day, has been found to be negligible. Many specimens had no measurable activity and only one specimen out of 30 had an activity as high as 10 units.

Rats, rabbits, cats and guinea pigs. Adult and newborn animals of these species have been investigated and the variations in the first 3 weeks of life are shown in Fig. 1. Several litters were used for the young of each species, and the points represent the mean of up to 8 specimens.

The following values had been found for the adult animals: rats, 13.1 units (mean of 6 males), rabbits, 12.2 and 11.5 units (2 males), cat, 24.0 units (1 female), guinea pigs, 40.0 and 57.0 units (2 females). The levels at birth were lower than those of the

adults in the rat and rabbit, and higher in the cat and guinea pig. Except in the guinea pig the general trend after birth was similar to that in man, in that there was a definite rise. The trend with the guinea pig was less certain.

The colostrum of one cat was investigated and found to contain less than 1 unit/ml.

Dogs The sera of 6 normal adults, 4 of whom were males, and 2 non pregnant females, averaged 38 units, and experiments have been made on 5 litters of puppies. The sera of puppies at birth had an activity of the same order of magnitude as the adults, but there was a rise in the first few days of life to about 800 units, and a fall in the next 2 weeks to about 50 units. Colostrum obtained from a bitch at the time of parturition was found to have an

activity of 6000 units, and this activity declined rapidly at first and then more slowly. The milk of one animal had an activity of 54 units, and of another 400 units, by the 5th day, and was still 20–30 units after a fortnight. Composite curves of the findings plotted on a logarithmic scale are shown in Fig 2.

The colostrum or milk expressible from glands which were not being suckled remained high after the activity in the milk from other glands had fallen to a lower level. Thus the activity of a pooled sample of fluid from unused glands on the 5th day was 918 units and on the 15th day was 2000 units.

The rise in the serum activity of the puppies depended upon the consumption of the mother's colostrum, for some puppies, which did not gain weight and thrive, presumably because they had not taken sufficient milk, had much smaller increases of activity in their sera than those which took milk

freely from birth and gained weight rapidly. Furthermore, puppies reared on evaporated cow's milk, which contained no measurable cholinesterase activity, showed no increase in serum activity although they grew as well (and their eyes opened at the same time) as litter mates kept with the mother. This is illustrated in Fig 2.

The activity of the bitches' serum did not rise very much post partum although there may have been a small increase in some instances. In one animal, the serum, before delivery, had an activity of 47 units. This had risen to 74 units on the following day and remained above 60 units for at least 17 days.

The enzyme present in bitches' milk hydrolysed benzoylcholine and acetylcholine but not acetyl β methylcholine. It therefore corresponds to the 'pseudo cholinesterase' of Mendel *et al* (1943).

DISCUSSION

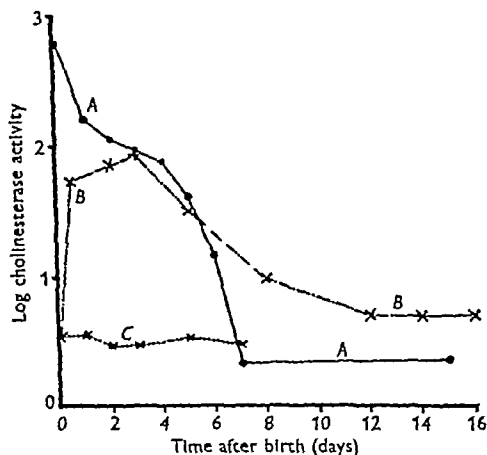


Fig 2 The cholinesterase activities of puppy sera and of bitches' milk during the first 16 days after birth. A, activity of mother's colostrum and milk, B, activity in the sera of puppies feeding from the mother, C, activity in the sera of puppies fed on evaporated cow's milk.

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puppy but would not appear to be essential since the three puppies reared on evaporated cow's milk thrived satisfactorily without it. Apart from this, however, bitches' colostrum should provide a valuable source of unspecific cholinesterase in high concentration, and it is hoped to investigate some of the problems arising out of the observations here presented.

SUMMARY

1 In newborn humans, rats, rabbits and cats the cholinesterase activity of the serum tended to be near the adult level and to rise soon afterwards to

about twice the initial value. In guinea pigs the activity did not rise.

2 The colostrum of man and of the cat had negligible cholinesterase activity.

3 In puppies the serum cholinesterase rose to 25 times its birth level in 3 days. This increase appears to be due to the very high cholinesterase activity of bitches' colostrum.

The human material was obtained from the Landes frauenklinik, Wuppertal, through the courtesy of the Director, Prof. Anselmino. A pure bred setter was obtained from Mr. Parry, the Canine Research Station, Kennet. Our thanks are also expressed to Miss M. F. Harrison for considerable assistance with work carried out at night.

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The Glucuronide-synthesizing System in the Mouse and its Relationship to β -Glucuronidase

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(Received 25 May 1949)

The β glucuronidase activity of mouse liver, kidney or uterus has been shown to reflect the state of proliferation of the tissue (Levy, Kerr & Campbell, 1948; Kerr, Campbell & Levy, 1949a). It would appear that β glucuronidase is distinct from the enzyme system responsible for the production of conjugated glucuronides in the body. The work of Lipschitz & Bueding (1939) suggests that this process is more complex than a simple condensation of the aglycone with free glucuronic acid, and that it takes place in liver and kidney only. β Glucuronidase, on the other hand, is present to a greater or less extent in practically all animal tissues that have so far been examined (see, for example, Oshima, 1934). Karunairatnam & Levy (1949) found that glucuronide synthesis by adult mouse liver slices was not appreciably impaired by saccharic acid in con-

centrations which caused almost complete inhibition of β glucuronidase.

The ability of various mouse tissues to synthesize glucuronides has been studied and compared with their glucuronidase activity under conditions leading to changes in the latter.

EXPERIMENTAL

Measurement of glucuronide synthesis. The conversion of *o*-aminophenol to its glucuronide in sulphate free bicarbonate Ringer solution was followed by the method of Levy & Storey (1949). Except in the case of lung, the tissue was sliced, and an amount corresponding to not less than 10 mg dry weight was taken for each estimation. If necessary, slices from more than one animal were pooled. Whenever possible, the estimation was done in quadruplicate in order to reduce the variable error in the procedure (see Levy & Storey, 1949). In the case of lung, the intact lobes were used. Boyland & McDonald (1948) have shown that this is permissible for measurements of metabolism in lung from young adult mice.

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Results are expressed in the tables as μg *o* aminophenol conjugated/g dry weight of tissue in 1 hr Both male and female mice were studied, but sex did not have any apparent effect on values for glucuronide synthesis, and it is therefore not usually shown in the tables

Glucuronidase activity The activity of this enzyme in tissue extracts was determined by the method of Kerr, Graham & Levvy (1948), and results are shown in terms of glucuronidase units (GU)/g moist tissue, where 1 GU liberates $1\mu\text{g}$ phenol in 1 hr from 0.015M phenylglucuronide at 37° and pH 5.2 In the case of tissues in which the kinetics of hydrolysis of phenylglucuronide by the enzyme have not yet been studied, it was assumed that optimal conditions for hydrolysis resemble those found for liver, spleen and kidney (Kerr *et al* 1948, Kerr *et al* 1949a)

RESULTS

Comparison of glucuronide synthesis and glucuronidase activity in various tissues Table 1 shows the glucuronide synthesizing power of liver, kidney, spleen and lung in young and adult mice, and of two

Table 1 The glucuronide synthesizing power and β glucuronidase activity of various mouse tissues

(When the mean is based on values for individual animals the standard error of the mean for the group is also shown Figures in brackets are numbers of animals used)

Tissue	Age of animal	<i>o</i> Aminophenol conjugated (μg /g dry weight)	Glucuronidase activity/g moist weight (GU)
Liver	Adult	570 ± 43 (41)	273 ± 13 (23)*
Kidney	Adult	150 ± 14 (4)	363 ± 24 (11)*
	9 days	30 (4)	—
	5 days	—	793 (4)*
Lung	Adult	Nil (2)	185 ± 19 (3)
	9 days	Nil (3)	316 ± 22 (3)
Spleen	Adult	Nil (3)	636 ± 70 (23)*
	9 days	Nil (4)	—
	5 days	—	3245 (4)*
Sarcoma (Crocker 180)	Adult	Nil (2)	433 ± 44 (6)
Carcinoma (Imperial Cancer Research Fund 2146)	Adult	Nil (2)	751 ± 75 (6)

* Quoted from Levvy *et al* 1948

transplantable mouse tumours In agreement with Lipschitz & Bueding (1939), the synthetic system was found only in liver and kidney, the latter being much the less active of the two tissues The ability of kidney slices to synthesize glucuronides was considerably less in young mice than in adults This was also true for liver, and Fig 1 shows the development of the synthetic system in the liver of the growing mouse

Also shown in Table 1 are figures for the glucuronidase activity of the various tissues, some quoted from earlier work from this Department, and others especially determined for present purposes The distribution of this enzyme obviously bears no relation to that of the synthetic system The higher glucuronidase activity of organs from young mice compared with those from normal adults, already

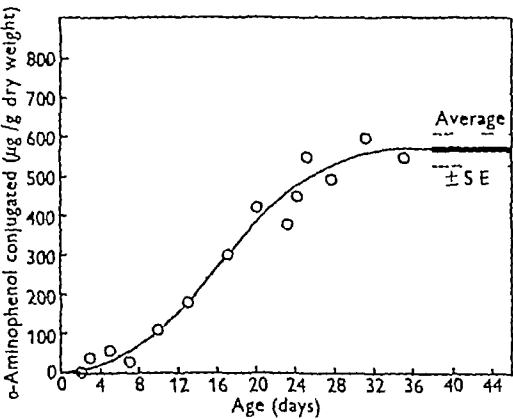


Fig 1 The development of the glucuronide synthesizing system, as measured by the conjugation of *o* amino phenol, in the liver of the growing mouse (own mixed colony)

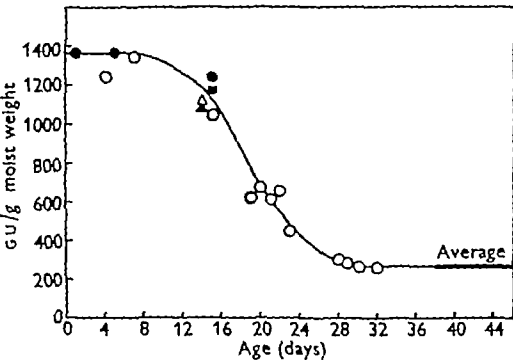


Fig 2 The change in glucuronidase activity in the liver of the growing mouse ■, strain C57, △, strain A, ●, strain CBA, ●, own mixed colony, August 1947, ○, own mixed colony, February 1949

noted for liver, kidney, spleen and uterus (Levy *et al* 1948, Kerr *et al* 1949a), is also seen in lung The change in liver glucuronidase activity has now been fully studied from birth until maturity, and the results are shown in Fig 2 This graph was compiled from results collected over a considerable period of time with four different strains of mice The glucuronidase activity of the young mice at a given age was remarkably constant for the different strains, and average figures for adults of different strains were indistinguishable from each other

In agreement with the observations of Fishman & Anlyan (1947) on human tissues, both mouse tumours were high in glucuronidase activity when compared with most normal adult tissues

The role of glucuronidase in glucuronide synthesis
The possibility had to be considered that the activity of the glucuronide synthesizing system in the liver does not vary with age, but that the hydrolytic activity of β glucuronidase at any given age determines the net amount of synthesis which can be measured (compare Figs 1 and 2). It has already been shown (Karunairatnam & Levvy, 1949) that saccharate in a concentration of $10^{-2}M$ causes almost complete inhibition of glucuronidase in liver or kidney extracts, but has no appreciable effect on glucuronide synthesis by adult mouse liver slices. In many of the experiments summarized in Table 1, additional determinations of glucuronide synthesis were made in presence of $10^{-2}M$ saccharate. This substance caused no increase in the synthetic power of infant liver or adult kidney, nor did its presence lead to glucuronide synthesis by lung, spleen or tumour slices. Failure of saccharate to penetrate the cell would appear to be excluded, since Campbell & Levvy (1949) have found that it inhibits hydrolysis of glucuronides by mouse kidney in the two histochemical tests of Friedenwald & Becker (1948). It seems, therefore, that the glucuronidase activity of a tissue has no bearing on its ability to synthesize glucuronides.

The effect of various measures on the glucuronide synthesizing system in vivo
Various measures leading to changes in the state of proliferation of mouse liver were examined for their action on the glucuronide synthesizing system. The results are shown in Table 2, and it can be seen that in no instance was there a detectable change in the activity of the synthetic system. Under similar conditions, a rise in glucuronidase activity to two or three times the normal value was seen after partial hepatectomy, or in jection of menthol, carbon tetrachloride or oestrone (Levy *et al* 1948, Kerr *et al* 1949a). Colchicine in the dose shown causes no change in the normal glucuronidase activity of liver, but it does prevent the rise in activity which follows such measures as partial hepatectomy. In the smaller dose, sorbic acid behaves like colchicine, but the larger dose (240 mg/kg) causes a profound depression in liver and kidney glucuronidase activity in normal mice. The effects of colchicine and sorbic acid on glucuronidase activity appear to be related to their actions as inhibitors of mitosis (Kerr, Campbell & Levvy, 1949b).

The effect of various compounds on the synthesis of o-aminophenylglucuronide in vitro
Four of the compounds examined for their action on the glucuronide synthesizing system *in vivo* were tested *in vitro* for their effect on the conversion of o-amino-

phenol ($0.00023M$) to its glucuronide by surviving liver slices from normal mice (Table 3). The fifth compound listed in Table 2, oestrone, was too sparingly soluble in water to permit its study in the present experiments.

Table 2 *The effect of various measures on the glucuronide synthesizing system in mouse liver*

(When the mean is based on values for individual animals the standard error of the mean for the group is also shown. Figures in brackets are numbers of animals used.)

Treatment	Dose (g/kg)	Days after treatment	o-Aminophenol conjugated/g dry weight (μg)
None	—	—	570 ± 43 (41)
(-) Menthol, intra peritoneally in olive oil	0.33	1 3 6	560 ± 40 (3) 640 (2) 650 (2)
Carbon tetrachloride, subcutaneously in olive oil	5.33	1 3 7	560 ± 170 (3) 640 ± 200 (3) 640 ± 150 (3)
Partial hepatectomy	—	3 7 10	670 ± 60 (6) 650 ± 50 (6) 610 ± 80 (6)
Oestrone, subcutaneously in olive oil (ovariectomized mice)	0.0017	4	580 ± 70 (3)
Colchicine, subcutaneously in aqueous solution	0.0015	1	450 ± 80 (6)
Sorbic acid, subcutaneously in aqueous solution	0.24 0.16	4 4	440 ± 70 (12) 540 ± 120 (6)

Table 3 *The effect of various compounds on the synthesis of o-aminophenylglucuronide by mouse-liver slices*

Compound	Concentration (M)	o-Aminophenol conjugated ($\mu g/g$ dry weight)		Inhibition (%)
		In controls	In presence of compound	
Sorbic acid	0.01	380	50	87
	0.01	970	370	62
	0.005	970	500	49
Colchicine	0.01	380	140	63
	0.01	530	190	64
	0.005	530	270	49
Carbon tetrachloride	0.0015	490	520	-6
	0.0015	660	520	21
(-) Menthol	0.001	450	120	73

Colchicine, sorbic acid and menthol were added as aqueous solutions (if necessary, after pH adjustment) during the preparation of the bicarbonate Ringer solution. In the case of CCl_4 , the Ringer solution was made saturated with the compound. In all experiments, controls were done with untreated slices from the same animal. Each determination was done in quadruplicate, and the standard error of the mean is thus about 10% (Levy & Storey, 1949).

As can be seen from Table 3, in the presence of colchicine, sorbic acid or menthol there was a drop in the conversion of *o*-aminophenol to its glucuronide which was outside the range of error. Inhibition was almost 50% with 0.005M colchicine or sorbic acid, and 73% with 0.001M menthol. Carbon tetrachloride had no appreciable effect in the highest concentration possible (0.0015M). None of the four compounds in question interfered in the colour reaction for *o*-aminophenylglucuronide.

DISCUSSION

From the results of the experiments described above, it seems clear that there are at least two distinct enzyme systems in the mouse concerned with the metabolism of the conjugated glucuronides. One of these, β -glucuronidase, is present in practically every tissue, and its action is probably entirely hydrolytic. The activity of this enzyme in a tissue varies with the degree of cell division in progress. The other enzyme system is responsible for the synthesis of glucuronides, and is probably complex. It has so far been found only in liver and, to a smaller extent, kidney. The activity of this enzyme system in liver is not altered by measures causing changes in the state of proliferation of the tissue. The ability of liver or kidney to synthesize glucuronides does, however, vary with the age of the animal. At birth, the activity of the synthetic system is small or nil, and it only reaches its ultimate value when the animals are 4 or 5 weeks old. Taken together, the two enzyme systems may provide a mechanism for regulating the transport, action and excretion of physiologically active, glucuronidogenic compounds, such as oestriol. Alternatively, their function may be to provide free glucuronic acid or a transformation

product for building up into more complex molecules.

Since menthol is known to form a glucuronide in the presence of surviving liver slices (Lipschitz & Bueding, 1939), its depressant action on the synthesis of *o*-aminophenylglucuronide *in vitro* is probably the result of competition with *o*-aminophenol rather than genuine inhibition of the synthetic mechanism. The inhibitory actions of sorbic acid and colchicine on glucuronide synthesis *in vitro* are difficult to interpret at present. The overall synthetic process is known to be adversely affected by other agents, such as cyanide, fluoride and iodoacetate (Lipschitz & Bueding, 1939), azide and sulphate (Dr I. D. E. Storey, private communication), but their mode of action is in most cases still obscure.

SUMMARY

1 The glucuronide synthesizing system in the mouse was found only in liver and, to a smaller extent, kidney.

2 Measures causing changes in the state of proliferation of liver had no effect on the activity of the glucuronide synthesizing system.

3 The activity of the synthetic system was almost nil at birth, and only reached its ultimate value when the mice were more than 1 month old.

4 The effects of various compounds on glucuronide synthesis *in vitro* were studied.

5 On the basis of this work, the enzyme system responsible for glucuronide synthesis can be clearly distinguished from β -glucuronidase.

The authors are indebted to Dr J. R. Riley for the supply of tumour-bearing mice, and to Mr D. Love for technical assistance. The expenses of this work were in part defrayed by a grant from the Medical Research Council.

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Chemical Studies of Peripheral Nerve During Wallerian Degeneration

1 LIPIDS

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(Received 27 May 1949)

When a peripheral nerve is cut, that portion of the nerve distal to the point of section soon loses its ability to transmit a nerve impulse. This is associated with a series of familiar histological changes known as Wallerian degeneration. Both histologists and histochemists have studied degenerating nerve in great detail, but in few instances have the techniques of chemistry been employed. Notable exceptions are the classical publications of Noll (1899), who observed that degenerating horse and dog nerves contained less 'protagon', and Mott & Halliburton (1901*a, b*), who reported the complete disappearance of phosphorus from degenerating cat nerves. These results, although of great interest, are of limited value because of the technical methods employed, usually a large sample of nerve tissue was needed and only one constituent of the nerve could be estimated from each sample. More recently, May (1930) has reported changes in the distribution of phosphorus and Abercrombie & Johnson (1946*b*) have described changes in the distribution of nitrogen, including collagen nitrogen, in degenerating rabbit nerves.

As the nerve degenerates, the lipid containing myelin sheath that surrounds the axon of each individual nerve fibre at first fragments and later is completely destroyed. Although the principal constituent of this sheath, 'myelin', is usually referred to as though it were a chemical entity, chemists have for a long time suspected it to be a mixture of substances. Recently, Johnson, McNabb & Rossiter (1948*a, b*, 1949*a*) have produced evidence that free cholesterol and the two sphingosine containing lipids or sphingolipids (cerebroside and sphingomyelin), rather than lecithin or cephalin, are the principal lipid components of the myelin sheath. They have called these lipids, i.e. free cholesterol, cerebroside and sphingomyelin, the myelin lipids.

For references to the extensive literature on the histological changes that occur during Wallerian degeneration the reader is referred to the publications of Ramon y Cajal (1928), Nageotte (1932), Weddell & Glees (1941), Young (1942), Holmes & Young (1942) and Weiss (1944). Since destruction of the myelin sheath is such an outstanding feature, it seemed of interest to investigate the changes in the

concentration of the myelin lipids in a degenerating nerve. Micromethods are now available that permit the determination of these and other lipids in small samples of nerve. A preliminary account of these experiments has already appeared (Johnson, McNabb & Rossiter, 1949*b*).

METHODS

The right sciatic nerve of 30 cats was cut at the level of the greater trochanter of the femur. The proximal stump was retracted and sutured to the overlying muscle. By separating the proximal and distal stumps in this way the possibility of regeneration was minimized. None of the animals showed either functional or post mortem evidence of regeneration. The operation was performed with full aseptic precautions under nembutal anaesthesia. No attempt was made to control the age, weight, or sex of the animals. After periods of time varying from 4 to 96 days the animals were killed and the distal degenerating segment of nerve removed. At the same time a similar length of left sciatic nerve was removed to serve as a control. Each nerve was cleaned of adherent fatty and epineural connective tissue and the lipids extracted with ethanol and ether as described previously (Johnson *et al.* 1948*a*). Samples of the ethanol ether extract were placed in a 60° water bath and evaporated just to dryness under reduced pressure and in an atmosphere of N₂. The lipids were extracted with light petroleum (b.p. 40–60°) and the phospholipins precipitated with acetone and MgCl₂. The precipitate was washed with acetone and then dissolved in a 1:1 methanol-ether mixture.

The concentration of cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin, lecithin, total fatty acid and neutral fat was determined in each nerve. From these figures the concentration of ester cholesterol, sphingomyelin, cephalin, essential lipid (i.e. all non triglyceride lipids), myelin lipid, and total lipid was calculated. All analyses were done in duplicate.

Analytical procedures

The analytical methods for cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin and lecithin have been described by Johnson *et al.* (1948*a*).

Cerebroside (glycosphingoside) was estimated in a sample of the original ethanol-ether extract by the method of Brand & Sperry (1941) in which galactose, liberated by acid hydrolysis, is estimated by the procedure of Miller & Van Slyke (1936). In this method, ferrocyanide, formed from the reduction of ferricyanide by the galactose, is titrated with ceric sulphate. Cerebroside was calculated as galactose $\times 4.5$.

Total and free cholesterol were estimated in the acetone soluble fraction by the method of Schoenheimer & Sperry (1934), incorporating improvements suggested by Sperry & Brand (1943) and Sobel & Mayer (1945).

Total phospholipin Total P was determined in a sample of the methanol-ether solution of the acetone-precipitated phospholipin by the method of King (1932). Total phospholipin was calculated as lipid P $\times 2.5$.

Monoaminophospholipin (phosphoglyceride) A sample of the methanol-ether solution of the acetone-precipitated phospholipins was hydrolysed in N KOH for 18 hr at 37° . Monoaminophospholipin was estimated as the total acid soluble P of the hydrolysate (Schmidt, Benotti, Hershman & Thannhauser, 1946).

Lecithin (phosphatidyl choline) was estimated as the acid soluble choline, determined by the method of Glick (1944), in the monoaminophospholipin hydrolysate (Haack, 1947). Lecithin was calculated as choline chloride $\times 5.58$.

Total fatty acid was determined in a sample of the original ethanol ether extract by a modification of the Bloor oxidative method described by Boyd (1938). After saponification and acidification, the free fatty acid and cholesterol were extracted with light petroleum (b.p. $40-80^\circ$) and oxidized by a measured quantity of $K_2Cr_2O_7$, excess of which was titrated with thiosulphate. After correction had been made for the cholesterol present, the total fatty acid was calculated on the assumption that 1 mg. of fatty acid was oxidized by 3.61 ml. 0.100N $K_2Cr_2O_7$ (Boyd, 1938).

Neutral fat An estimate of triglyceride was obtained by determining the glycerol in a portion of the phospholipin free acetone solution by the method of Vornis, Ellis & Maynard (1940). After saponification and acidification, the fatty acids and cholesterol were removed by extraction with light petroleum (b.p. $40-80^\circ$) and the glycerol allowed to react with a measured quantity of potassium periodate. Excess periodate was titrated with thiosulphate. The periodate used gave a measure of the glycerol in the sample. Neutral fat, based on an assumed mean molecular weight of 283 for the fatty acids, was calculated as glycerol $\times 9.64$.

In addition, a value for neutral fat was derived from the figures for neutral fat fatty acid, i.e. the difference between the total fatty acid and the sum of the fatty acids of cerebroside, cholesterol ester, lecithin, sphingomyelin and cephalin. In the absence of detailed information concerning the nature of the fatty acids of the lipids of nerve,* the figures were calculated on the basis of an assumed mean molecular weight of 368, that of lignoceric acid, for the fatty acids of the two sphingolipids, cerebroside and sphingo-

myelin, and an assumed mean molecular weight of 283 for the fatty acids of the phosphoglycerides and cholesterol ester. A molecular weight differing from the assumed mean for the fatty acids of either the sphingolipids or the phosphoglycerides would not appreciably alter the general conclusions. The neutral fat fatty acids were, therefore, calculated as total fatty acid (cerebroside $\times 0.45$ + ester cholesterol $\times 0.73$ + lecithin $\times 0.70$ + sphingomyelin $\times 0.44$ + cephalin $\times 0.74$). Based on an assumed mean of 283 for the triglyceride fatty acids, the neutral fat was calculated from the neutral fat fatty acid by multiplying by the factor 1.045.

Ester cholesterol was calculated as the difference between the total cholesterol and the free cholesterol.

Sphingomyelin (phosphosphingoside) was calculated as the difference between the total phospholipin and the monoaminophospholipin.

Kephalin, representing all the non choline containing monoaminophospholipins, was calculated as the difference between the monoaminophospholipin and lecithin.

Essential lipid, representing the non triglyceride lipid, was the sum of cerebroside, total cholesterol and total phospholipin.

Myelin lipid was the sum of cerebroside, free cholesterol and sphingomyelin.

Total lipid was the sum of the essential lipid and neutral fat.

Specificity of analytical methods

The specificity of a micromethod, when it is usually impossible to characterize fully the substance to be estimated, is frequently far from absolute. Although we feel that, for the most part, we have estimated the substance for which each method was designed, it is important that possible deficiencies of the methods should not be forgotten. For instance, any substance, other than the known cerebroside, that is soluble in the solvents used and from which reducing substances are released on acid hydrolysis, would be estimated as cerebroside. A similar qualification applies to the method for cholesterol. Although this method can now be described as standard, it is possible that small quantities of other digitonin precipitable substances that give the Liebermann Burchard colour reaction would be determined as cholesterol.

The method for total phospholipin is also standard, but that for monoaminophospholipin is less so. In this method known monoaminophospholipins, such as lecithin (phosphatidyl choline) and the cephalins (phosphatidyl ethanolamine, phosphatidyl serine and brain phosphomositide), would be measured together with any other unknown easily hydrolysed phospholipins. If such substances contained choline in a form readily liberated on hydrolysis, they would be estimated as lecithin, if, as seems more likely, such unknown phospholipins contained no easily hydrolysed choline, they would be estimated as cephalin. In degenerating nerve, however, it is possible that choline containing degradation products of either sphingomyelin or lecithin may be present. If such a substance were both soluble in light petroleum and insoluble in acetone, it would be estimated as lecithin. One possible degradation product of lecithin, β glycerylphosphorylcholine, studied by Schmidt, Hershman & Thannhauser (1945), is insoluble in light petroleum, and so would not be present in the phospholipin extracts. Since sphingomyelin is measured as the difference between the total P and the readily hydrolysed P of the

* See, however, Chibnall, Piper & Williams (1936), who found that lignoceric acid from the brain cerebroside, lécasin, was a mixture of n heneicosane-, n tricosane- and n pentacosane-1 carboxylic acids, whilst phrenosinic acid from the brain cerebroside, phrenosin, was a mixture of 2 hydroxy n heneicosane-, n tricosane- and n pentacosane-1 carboxylic acids.

lipid extract, unknown phospholipins, not easily hydrolysed, would be determined as sphingomyelin

The method for total fatty acid is also not specific, and, in addition to cholesterol, for which correction is made, other non saponifiable lipids may be measured. The sphingolipids are difficult to saponify and, even if saponification is complete, part of the liberated sphingosine may pass into the light petroleum together with the fatty acids and cholesterol. This may account for the difference in the figures for neutral fat calculated from neutral fat fatty acid and those calculated from glycerol (some 30% for the control nerves). Also the glycerol of any degradation product of either triglyceride or phosphoglyceride that is soluble in light petroleum and not precipitated by acetone would be measured by the methods used.

Accuracy of analytical methods

The coefficient of variation of the overall procedure for each of the individual lipids was as follows: cerebroside $\pm 3.5\%$, free cholesterol $\pm 0.0\%$, total cholesterol $\pm 0.9\%$, total phospholipin $\pm 1.3\%$, monoaminophospholipin $\pm 1.6\%$, lecithin $\pm 5.1\%$, total fatty acid $\pm 3.1\%$, and neutral fat $\pm 3.1\%$. The accuracy of the methods for those lipids derived by difference would usually be less. Since the mean of duplicate estimations was always recorded, the probable error of each observation was less than the above figure.

Recording of results

For the first 32 days after the operation the wet weight of the degenerating nerve was greater than that of the same length of nerve from the control side. This presented the

problem of how best to record the results. To express the results in terms of unit wet weight of the degenerating nerve would be misleading, while to express them in terms of unit dry weight of the degenerating nerve would also be unsatisfactory, for the degenerating nerve contained much less lipid than the normal nerve, and lipid accounts for a high percentage of the dry weight of nerve. Therefore we have expressed all results for degenerated nerve in terms of the wet weight of the same length of control nerve from the opposite side. This is equivalent to the fresh weight of the degenerating nerve before it was sectioned, i.e. at zero time.

RESULTS

Animals were killed at intervals of 4, 8, 16, 32, 64 and 96 days after the nerve section. Table 1 gives the mean and the standard error of the mean for the concentration of cerebroside, total, free and ester cholesterol, total phospholipin and essential lipid in the control and degenerated nerves. The table also gives the value of *P* obtained for each lipid in testing the significance of the difference between the means for the degenerated and control nerves.

The concentration of cerebroside, 2.4 mg/100 mg for the control nerves, changed little during the first 8 days of degeneration and then decreased rapidly, reaching 0.6 mg/100 mg, or 25% of the total, by 32 days. The concentration of cerebroside then decreased more gradually, no measurable cerebroside remaining by 96 days.

Table 1 *Lipids of cat nerve during Wallerian degeneration*

(mg/100 mg wet wt. of control nerve)

No. of animals	Degenerated											
	Control 30			(4 days) 5			(8 days) 7			(16 days) 5		
	Mean	S.E.M.		Mean	S.E.M.	<i>P</i>	Mean	S.E.M.	<i>P</i>	Mean	S.E.M.	<i>P</i>
Cerebroside	2.40	± 0.11		2.48	± 0.32	>0.7	2.30	± 0.23	>0.6	1.32	± 0.12	<0.01
Total cholesterol	3.21	± 0.05		3.22	± 0.16	>0.9	3.09	± 0.15	>0.3	2.66	± 0.19	<0.01
Free cholesterol	3.19	± 0.05		3.17	± 0.16	>0.8	2.92	± 0.13	<0.05	1.66	± 0.06	<0.01
Ester cholesterol (total - free cholesterol)	0.02	± 0.02		0.04	± 0.02	>0.2	0.17	± 0.04	<0.01	1.00	± 0.17	<0.01
Total phospholipin	6.04	± 0.13		6.12	± 0.26	>0.8	6.00	± 0.55	>0.9	2.98	± 0.13	<0.01
Essential lipid (cerebroside + total cholesterol + total phospholipin)	11.63	± 0.26		11.82	± 0.56	>0.3	11.43	± 0.87	>0.7	6.96	± 0.29	<0.01

No. of animals	Degenerated											
	Control 30			(32 days) 5			(64 days) 5			(96 days) 3		
	Mean	S.E.M.		Mean	S.E.M.	<i>P</i>	Mean	S.E.M.	<i>P</i>	Mean	S.E.M.	<i>P</i>
Cerebroside	2.40	± 0.11		0.60	± 0.16	<0.01	0.48	± 0.11	<0.01	0	± 0	<0.01
Total cholesterol	3.21	± 0.05		1.94	± 0.19	<0.01	1.02	± 0.05	<0.01	0.77	± 0.05	<0.01
Free cholesterol	3.19	± 0.05		1.16	± 0.05	<0.01	0.54	± 0.06	<0.01	0.29	± 0.05	<0.01
Ester cholesterol (Total - free cholesterol)	0.02	± 0.02		0.79	± 0.35	<0.01	0.48	± 0.07	<0.01	0.47	± 0.08	<0.01
Total phospholipin	6.04	± 0.13		1.30	± 0.13	<0.01	0.74	± 0.05	<0.01	0.60	± 0	<0.01
Essential lipid (cerebroside + total cholesterol + total phospholipin)	11.63	± 0.26		3.86	± 0.26	<0.01	2.22	± 0.14	<0.01	1.37	± 0.04	<0.01

The concentration of total cholesterol, 3.21 mg/100 mg in the control nerves, changed little during the first 8 days and then decreased steadily during the course of the degeneration, 0.77 mg/100 mg still remaining after 96 days. On the other hand, free cholesterol, which accounted for almost all of the cholesterol of the control nerves, decreased to 2.92 mg/100 mg ($P < 0.05$) after 8 days. From 8 to 32 days the concentration of free cholesterol fell rapidly, and thereafter more slowly, only 0.29 mg/100 mg remaining after 96 days. There was virtually no ester cholesterol in the control nerves and during the period 0-8 days the concentration increased slowly, reaching 0.17 mg/100 mg ($P < 0.01$) by the 8th day. The concentration of ester cholesterol then increased rapidly, reaching a maximum of 1.0 mg/100 mg in 16 days. After 16 days the concentration decreased, but more slowly than the concentration of free cholesterol, so that by 96 days more of the cholesterol was in the ester form than in the free.

The concentration of total phospholipin, which was 6.04 mg/100 mg for the control nerves, also changed little during the first 8 days. It then decreased rapidly between 8 and 32 days and thereafter

more slowly, only 0.6 mg/100 mg, or 10% of the total, remaining after 96 days. The concentration of essential lipid, representing the sum of the concentrations of cerebroside, total cholesterol and total phospholipin, was 11.63 mg/100 mg in the control nerves. This decreased in a manner similar to the total phospholipin, only somewhat more slowly. By 96 days 1.37 mg/100 mg essential lipid remained, or 12% of the control value.

Table 2 gives the figures for the individual phospholipins, viz. lecithin, sphingomyelin and cephalin, and also those for monoaminophospholipin, total phospholipin and myelin lipid. The concentration of none of these substances changed appreciably during the first 8 days. Between 8 and 32 days the concentration of total phospholipin, sphingomyelin and myelin lipid decreased rapidly and after 32 days more slowly, 10, 7 and 6% respectively remaining after 96 days. Cephalin, the concentration of which was 2.97 mg/100 mg in the control nerves, disappeared very rapidly between 8 and 32 days, 0.28 mg/100 mg, or only 9% of the total, remaining at the end of this time. Subsequently, the concentration of cephalin did not change greatly. Lecithin, which

Table 2 *Phospholipins of cat nerve during Wallerian degeneration*

(mg/100 mg wet wt. of control nerve)

No of animals	Degenerated											
	Control 30		(4 days) 5			(8 days) 7			(16 days) 5			
	Mean	S.E.M	Mean	S.E.M	P	Mean	S.E.M	P	Mean	S.E.M	P	
Total phospholipin	6.04	±0.13	6.12	±0.26	>0.8	6.00	±0.55	>0.8	2.98	±0.13	<0.01	
Monoaminophospholipin	3.70	±0.14	4.08	±0.43	>0.2	3.84	±0.28	>0.6	1.60	±0.12	<0.01	
Lecithin	0.73	±0.04	0.80	±0.05	>0.4	0.82	±0.06	>0.2	0.51	±0.10	<0.05	
Sphingomyelin	2.33	±0.12	2.00	±0.39	>0.3	2.19	±0.40	>0.6	1.36	±0.12	<0.01	
(Total phospholipin - monoaminophospholipin)												
Kephalin	2.97	±0.13	3.32	±0.46	>0.3	3.01	±0.26	>0.8	1.12	±0.15	<0.01	
(Monoaminophospholipin - lecithin)												
Myelin lipid	7.91	±0.21	7.72	±0.69	>0.7	7.43	±0.70	>0.3	4.34	±0.16	<0.01	
(Cerebroside + free cholesterol + sphingomyelin)												
No of animals	Degenerated											
	Control 30		(32 days) 5			(64 days) 5			(96 days) 3			
	Mean	S.E.M	Mean	S.E.M	P	Mean	S.E.M	P	Mean	S.E.M	P	
Total phospholipin	6.04	±0.13	1.30	±0.13	<0.01	0.74	±0.05	<0.01	0.60	±0	<0.01	
Monoaminophospholipin	3.70	±0.14	0.66	±0.09	<0.01	0.48	±0.04	<0.01	0.40	±0	<0.01	
Lecithin	0.73	±0.04	0.36	±0.03	<0.01	0.24	±0.03	<0.01	0.18	±0.03	<0.01	
Sphingomyelin	2.33	±0.12	0.66	±0.13	<0.01	0.24	±0.07	<0.01	0.17	±0.04	<0.01	
(Total phospholipin - monoaminophospholipin)												
Kephalin	2.97	±0.13	0.28	±0.08	<0.01	0.24	±0.02	<0.01	0.23	±0.04	<0.01	
(Monoaminophospholipin - lecithin)												
Myelin lipid	7.91	±0.21	2.40	±0.27	<0.01	1.28	±0.17	<0.01	0.50	±0.06	<0.01	
(Cerebroside + free cholesterol + sphingomyelin)												

was present to the extent of only 0.73 mg/100 mg in the control nerves, decreased less rapidly than either the total phospholipin or myelin lipid.

Table 3 gives the figures for total fatty acid, neutral fat calculated from glycerol, neutral fat calculated from neutral fat fatty acid, and total lipid.

fat estimated as glycerol and that calculated from neutral-fat fatty acid. Since the figure for neutral fat fatty acid was derived by difference and includes errors, not only in the total fatty acid procedure, but also those in the estimation of phospholipin, cholesterol and cerebroside, it can be considered as little

Table 3 *Fatty acids and neutral fat of cat nerve during Wallerian degeneration*

(mg/100 mg wet wt. of control nerve. No. of animals is stated in parentheses under each result.)

	Control		Degenerated								
			(4 days)			(8 days)			(16 days)		
	Mean	S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Total fatty acid	14.54 (20)	±0.50	10.30 (3)	±1.13	<0.01	11.26 (5)	±1.00	<0.01	10.48 (4)	±0.83	<0.01
Neutral fat (calculated from glycerol)	6.91 (19)	±0.44	4.41 (4)	±0.76	<0.05	3.65 (4)	±0.27	<0.01	6.22 (3)	±0.88	>0.5
Neutral fat (calculated from neutral fat fatty acid)	10.14 (20)	±0.60	5.24 (3)	±1.00	<0.01	6.60 (5)	±0.59	<0.01	7.78 (4)	±0.94	>0.1
Total lipid (Essential lipid + neutral fat)	18.36 (19)	±0.40	16.00 (4)	±1.11	<0.05	14.52 (4)	±1.65	<0.01	12.80 (3)	±1.09	<0.01

	Control		Degenerated								
			(32 days)			(64 days)			(96 days)		
	Mean	S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Total fatty acid	14.54 (20)	±0.50	12.05 (2)	±2.86	>0.1	10.57 (3)	±0.54	<0.01	10.37 (3)	±2.33	<0.02
Neutral fat (calculated from glycerol)	6.91 (19)	±0.44	7.49 (2)	±3.97	>0.7	9.58 (3)	±0.57	<0.05	6.61 (2)	±1.69	>0.8
Neutral fat (calculated from neutral fat fatty acid)	10.14 (20)	±0.60	11.02 (2)	±2.94	>0.6	9.85 (3)	±0.58	>0.8	10.10 (3)	±2.37	>0.9
Total lipid (Essential lipid + neutral fat)	18.36 (19)	±0.40	11.35 (2)	±3.35	<0.01	11.87 (3)	±0.59	<0.01	7.95 (2)	±1.75	<0.01

The concentration of total fatty acid fell from 14.54 mg/100 mg to 10.30 mg/100 mg in 4 days ($P < 0.01$) and remained close to this figure through out the course of the degeneration. For each of the periods studied the difference between the values for the degenerating and control nerves was statistically significant, except for the 32 day nerves where fatty acid figures were available for two animals only. It is likely that, had the group been larger, this figure also would have been significant.

The concentration of neutral fat estimated as glycerol was 6.91 mg/100 mg in the control nerves. This fell to 4.41 mg/100 mg after 4 days ($P < 0.05$) and 3.65 mg/100 mg after 8 days ($P < 0.01$). The value for neutral fat calculated from neutral fat fatty acid was 10.14 mg/100 mg for the control nerves, falling to 5.24 mg/100 mg after 4 days ($P < 0.01$) and 6.6 mg/100 mg after 8 days ($P < 0.01$). After 8 days the concentration of neutral fat determined by either method did not differ significantly from that of the control nerves. There is an obvious discrepancy between the concentration of neutral

more than an approximation. It is reassuring to note that, despite the differences in the values for neutral fat obtained by the two methods, the general trend of the figures was the same for each. Both methods demonstrated the initial fall in the concentration of neutral fat after 4 and 8 days, followed by a return to normal values.

The concentration of total lipid was 18.36 mg/100 mg for the control nerve and decreased steadily throughout the course of the degeneration, reaching 7.95 mg/100 mg, or 43% of the total, after 96 days.

In Table 4 the figures for each lipid are expressed as a percentage of the figure for the control nerve of the opposite side, except for ester cholesterol where the figure is expressed as a percentage of the highest value, that of the 16th day. By presenting the data from Tables 1-3 in this manner it is possible to appreciate the rate at which each lipid disappears.

Table 4 also shows the wet weight of the degenerating nerve expressed as a percentage of the weight of the control nerve. The wet weight was greatest after 4 days ($P < 0.01$), but the figures for the wet

Table 4 *Lipids of cat nerve during Wallerian degeneration*

(Concentrations expressed as a percentage of the control nerve concentration, except for ester cholesterol)

	Control	Degenerated					
		4 days	8 days	16 days	32 days	64 days	96 days
Cerebroside	100	103	96	55	25	20	0
Total cholesterol	100	100	96	83	60	32	24
Free cholesterol	100	99	92	52	36	17	9
Ester cholesterol (expressed as a percentage of 16-day concentration)	2	4	17	100	79	48	47
Total phospholipin	100	101	99	49	22	12	10
Monoaminophospholipin	100	110	104	43	18	13	11
Lecithin	100	110	112	70	49	33	25
Sphingomyelin	100	86	94	58	28	10	7
Kephalin	100	112	101	38	9	8	8
Essential lipid	100	102	98	60	33	19	12
Myelin lipid	100	98	94	55	30	16	6
Total fatty acid	100	71	77	72	83	73	71
Neutral fat (calculated from glycerol)	100	64	53	90	108	139	96
Neutral fat (calculated from neutral fat fatty acid)	100	52	65	79	109	97	100
Total lipid	100	87	79	70	62	65	43
Wet weight	100	134	122	126	120	105	90

weight after 8 ($P < 0.01$), 16 ($P < 0.02$) and 32 days ($P < 0.05$) were all significantly greater than those of the control nerves. After 32 days the difference between the wet weight of the degenerating and the control nerves was not statistically significant.

DISCUSSION

Previous observations The increase in the wet weight of degenerating nerve was reported by Mott & Halliburton (1901a, b) and confirmed by May (1930) and Abercrombie & Johnson (1946b).

Our findings on the changes in the concentration of lipids agree well with those of previous workers. Noll (1899) reported a decrease in the concentration of 'protagon' in degenerating horse and dog nerves. It is unfortunate that this excellent work was done under the shadow of the 'protagon' controversy. There can be no doubt that 'protagon', once believed to be the mother of all brain substances, contributed greatly to our present ignorance of the chemistry of 'myelin'. Excellent accounts of this interesting controversy are those of Posner & Gies (1905) and MacLean (1918).

Noll (1899) estimated 'protagon' in nerves by the reducing substances liberated during acid hydrolysis. For the most part, he was measuring galactose liberated from the cerebroside. He reported that the total solid of an ethanol extract of a dog nerve, which had degenerated for 15 days, decreased less than the phosphorus of the extract, i.e. that the phospholipin disappeared from the nerve more rapidly than the total lipid. He also reported that 'protagon', probably cerebroside, of another nerve, which had degenerated for 16 days, decreased to 54% of the value for the control nerve. This result can be compared

with our mean cerebroside concentration of 55% for 16 day cat nerves.

Mott & Halliburton (1901a, b) reported that the concentration of total phosphorus in a degenerating cat nerve fell to zero in 29 days. The method for phosphorus estimation must have been extremely insensitive, for our experiments indicate that considerable phospholipin remains in the nerve after 32 days. May (1930) also found a decrease in total phosphorus in degenerating rabbit nerves, greatest between the 7th and 49th day. There was still 35% of the total phosphorus present even after 100 days. He also reported a rapid decrease of lipid P after 7 days and a considerable increase in water soluble P throughout the whole period of the degeneration.

The composition of 'myelin' While studying the chemical nature of the lipids of 'myelin', Johnson *et al* (1948b) found that the distribution of lipids in peripheral nerve resembled that of the lipids of the white matter of the brain rather than that of the grey matter. Brain white matter and peripheral nerve, both of which are rich in myelinated fibres, differed from brain grey matter in that they contained more cerebroside, free cholesterol and sphingomyelin (Johnson *et al* 1948a). Subsequently, Johnson *et al* (1949a) reported that it was these same three lipids, cerebroside, free cholesterol and sphingomyelin, that distinguished the white matter of the adult brain from that of the brain of the newborn infant, where myelination is incomplete. It was suggested that these three lipids, i.e. free cholesterol and the two sphingolipids, cerebroside (or glycosphingoside) and sphingomyelin (or phosphosphingoside), rather than the phosphoglycerides, lecithin and kephalin, were the principal lipid components of the myelin sheath.

These lipids were called the myelin lipids. Carter, Haines, Ledyard & Norris (1947) found a high concentration of the sphingolipids, cerebroside and sphingomyelin, in ox spinal cord.

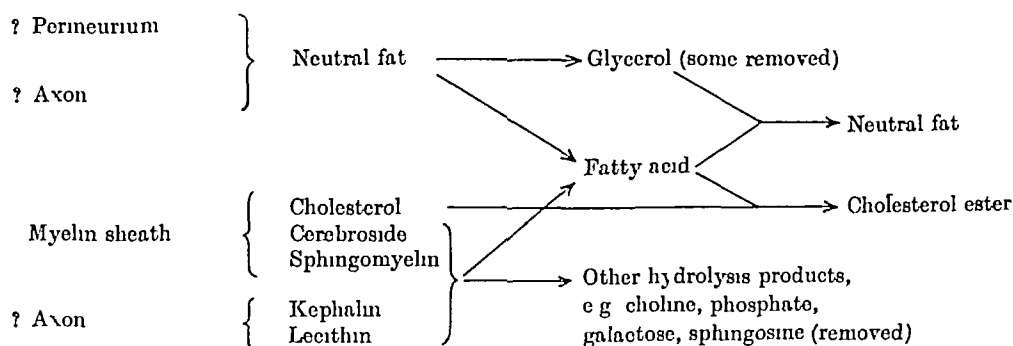
Each of the myelin lipids decreased in the degenerating nerve at approximately the same rate and to the same extent (Table 4). The change in the concentration of none of the other lipids resembled that of the myelin lipids, total lipid decreased much less rapidly, neutral fat decreased early, and then returned to normal, total cholesterol decreased more slowly, cholesterol ester, not present in normal nerve, appeared during the course of the degeneration, total phospholipid decreased at first more rapidly and then more slowly than the myelin lipid, lecithin decreased much more slowly and kephalin more rapidly (Table 7). The finding that cerebroside, free cholesterol and sphingomyelin decreased at the same rate and to the same extent is additional evidence supporting our previous suggestion that these substances, rather than lecithin and kephalin, are the principal lipid constituents of the myelin sheath.

Frequently the essential lipids are regarded as the important structural lipids of the body. Because a large proportion of the essential lipid of nerve is myelin lipid, they both tend to disappear from a degenerating nerve at a similar rate (Table 4). Although the essential lipids are important elements of the central nervous system as a whole, the myelin lipids would appear to play a more important role in the structure of the myelin sheath.

1939, Schmitt, Bear & Palmer, 1941). These studies indicate that the myelin sheath consists of coaxial concentric sheets of oriented lipid molecules alternating with thin, possibly unimolecular, layers of protein. The sheath shows a characteristic birefringence with the optical axes directed radially and a definite X-ray diffraction pattern. Such valuable studies tell us little, however, of the chemical nature of the lipid in this lipid-protein complex. It is suggested that this complex is rich in free cholesterol and the sphingosine-containing lipids, cerebroside and sphingomyelin.

Lipids of myelin during Wallerian degeneration

We can now give an outline of some of the changes that occur in the lipids when a peripheral nerve undergoes Wallerian degeneration. During the early stages the water content of the nerve increases, the absolute amount of the neutral fat decreases and there is little change in the myelin lipids. From 8 to 32 days there is a steady decrease in the concentration of the myelin lipids, i.e. cerebroside, free cholesterol and sphingomyelin. These substances are presumably slowly hydrolysed and the products of hydrolysis, e.g. glycerol, fatty acid, choline, galactose, sphingosine and phosphate, are removed. There is, in addition, a rapid hydrolysis of kephalin and a slower hydrolysis of lecithin. Some of the fatty acids, liberated during the hydrolysis, may combine with free cholesterol to form cholesterol ester and others may be converted into neutral fat. These changes can be represented schematically as follows.



Abercrombie & Johnson (1946b) found that most of the non-extractable nitrogen of rabbit nerve disappeared at a time similar to that during which we have observed the disappearance of the myelin lipid in cat nerve. This non-extractable nitrogen probably represents the so-called 'neurokeratin' (Block, 1937) and may be an important structural element of the myelin sheath.

Hitherto most of our knowledge of the constitution of the myelin sheath has come from polarized light and X-ray diffraction studies (Schmitt & Bear,

It should be stressed that the analyses were performed on the whole nerve and therefore represent not only the lipids of myelin, but also those of the axon, Schwann cells, macrophages, perineural and endoneurial connective tissue and, perhaps, some epineurial connective tissue also. The neutral fat is probably chiefly in the connective tissue, but some may be in the axon. The high proportion of kephalin and lecithin in grey matter of brain (Johnson *et al* 1948a, 1949a) would suggest that these lipids are chiefly constituents of the axon.

A scheme, admittedly hypothetical, representing the demyelination process was given by Page (1937). The chief experimental evidence, quoted in support of the scheme, was the autolysis experiments of Jungmann & Kimmelstiel (1929) and Backlin (1930). The former workers found that cerebroside decreased and inorganic phosphorus increased when rabbit whole brain stood in either oxygen or nitrogen. Backlin (1930) described a fall in the concentration of free cholesterol and cerebroside, and a possible increase in the concentration of phospholipin when a rabbit brain stood for 24 hr. However, he presented the results of one experiment only. Johnson, McNabb & Rossiter (1949c) found no change in the concentration of cerebroside or total cholesterol in slices of cat brain which were incubated in a buffer for periods of time up to 14 days.

The text book description of the chemistry of demyelination, that 'myelin' is composed of phospholipin which is converted into triglyceride, is probably derived from the views of Mott & Halliburton (1901a, b). That phospholipin is converted into triglyceride may be true in part, but phospholipin hydrolysis and triglyceride formation are only two of a number of changes that occur. In the past, undue emphasis has been placed on these two processes (Mott & Barratt, 1899, Setterfield & Sutton, 1935).

The period of greatest destruction of 'myelin' (8-32 days) is characterized by a decrease in the concentration of the myelin lipids, an increase in the concentration of ester cholesterol, and a return to normal of the concentration of neutral fat. During this time macrophages appear along the length of the degenerating nerve and there is a great proliferation of the Schwann cells (Holmes & Young, 1942, Young, 1942, Abercrombie & Johnson, 1946a). It is possible that either the macrophages or the Schwann cells contribute enzymes to aid the degradation of the lipids. The histological evidence suggests that quite large pieces of organized 'myelin' are engulfed by the macrophages and subsequently degraded. The 'myelin' is most likely destroyed while the macrophages are in the degenerating nerve, although the possibility that the macrophages remove themselves and destroy the 'myelin' elsewhere cannot be excluded. The remarkable similarity of the disappearance curves for each of the individual myelin lipids would favour the theory of particulate digestion followed by rapid hydrolysis or removal. Johnson *et al.* (1949c) showed that, when brain tissue was autolysed *in vitro*, phospholipin, chiefly cephalin and sphingomyelin, was destroyed, but that there was no destruction of cerebroside or cholesterol.

This would indicate that the *in vitro* degradation of the myelin lipids is different from their *in vivo* degradation. Possibly enzymes, other than those present in the non degenerating nervous system, e.g. those of the macrophages or the Schwann cells, are necessary for complete breakdown of the myelin lipids.

Our findings are also of interest because the quantitative changes in the concentration of lipids in a degenerating nerve can be correlated with the results of previous physiological and biological investigations of Wallerian degeneration. A discussion of our results in the light of such studies will have to be postponed for a subsequent paper.

SUMMARY

1 The concentration of cerebroside, total and free cholesterol, total phospholipin, monoamino phospholipin, lecithin, total fatty acid and neutral fat was determined in degenerating cat sciatic nerves at intervals of time from 4 to 96 days after nerve section. From these figures the concentration of ester cholesterol, sphingomyelin, cephalin, essential lipid, myelin lipid, and total lipid was calculated.

2 The wet weight of the degenerating nerve increased rapidly, reaching a maximum in 4 days and returning to normal after 64 days.

3 The total lipid content of the nerve decreased steadily throughout the course of the degeneration.

4 Neutral fat decreased rapidly, reaching a minimum between 4 and 8 days, and returned to normal by 32 days.

5 The myelin lipids (cerebroside, free cholesterol and sphingomyelin) changed little during the first 8 days and then decreased rapidly, and to the same extent, between 8 and 32 days.

6 Cholesterol changed little during the first 8 days. Between 8 and 32 days free cholesterol decreased rapidly and ester cholesterol, absent in control nerves, increased, reaching a maximum by 16 days.

7 Total phospholipin, which changed little during the first 8 days, decreased rapidly between 8 and 32 days. Sphingomyelin decreased at a similar rate, whereas cephalin decreased more rapidly and lecithin more slowly.

8 The results are discussed with reference to the chemical nature of the lipids of 'myelin' and to the changes that occur in the lipids when a nerve undergoes Wallerian degeneration.

Thanks are due to Mr Norval Burt and Miss Ann Boyce for skilful technical assistance. The work was financed by a grant from the National Research Council of Canada.

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Determination of *p*-Aminosalicylic Acid as *m*-Aminophenol

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(Received 30 May 1949)

Several methods have been described for the estimation of *p* aminosalicylic acid (4 amino 2 hydroxybenzoic acid) in blood (Lehmann, 1946, Ragaz, 1948, Tennent & Leland, 1948, 1949, Klyne & Newhouse, 1948) None of the procedures, developed in connexion with therapeutic trials of this drug in tuberculous, were micromethods, our aim was to develop a method suitable for use with capillary blood Bratton & Marshall's (1939) estimation of sulphonamides in blood seemed an obvious choice Such a method was used (but not described) by McClosky, Smith & Frias (1948) These workers recorded erratic results with *p* aminosalicylic acid, but obtained quantitative linear curves by allowing

solutions of the drug to stand for several days at room temperature, or by heating them at 100° for 30–60 min

Aqueous solutions of *p* aminosalicylic acid are known to be decarboxylated on standing or heating (Venkataraman, Venkataraman & Lewis, 1948, Rosdahl, 1948, Oberweiger, Seymour & Simmonite, 1948) The findings of McClosky *et al* (1948) could be explained as the decarboxylation of *p* amino salicylate followed by diazotization of *m* amino phenol We thought that such a technique could be used for the micro-estimation of *p* aminosalicylic acid and set about establishing conditions for complete decarboxylation

While this work was in progress four diazotization methods were described. One is an adaptation by Marshall (1948) of the original Bratton & Marshall (1939) procedure, on which the two other methods (Way, Smith, Howie, Weiss & Swanson, 1948, Newhouse & Klyne, 1949) are also based. In the fourth method (Desbordes & Henry, 1948) diazotized *p* aminosalicylic acid is coupled with thymol.

EXPERIMENTAL

Bratton & Marshall's procedure for estimating total sulphonamide in blood was progressively modified, and two alternative methods, *A* and *B*, were finally adopted.

Reagents Trichloroacetic acid (20% w/v in water) H_2SO_4 (21.5 \pm 0.5N, made by adding 60 ml conc H_2SO_4 to 40 ml water, titrating and readjusting the volume) NaOH (8N) NaNO_2 (1% w/v in water) Ammonium sulphamate (5% w/v in water) *N* 1 Naphthylethylenediamine dihydrochloride (0.1% in water) Ethanol (95%) or industrial methylated spirit.

Standard solutions (1) Stock standard equivalent to 100 mg *p* aminosalicylic acid/100 ml. 137.9 mg pure sodium *p* aminosalicylate dihydrate is dissolved in 100 ml water, or 71.2 mg pure *m* aminophenol is dissolved in 100 ml 0.01N HCl. These can be kept in colourless bottles at room temperature for at least 8 weeks. (2) Dilute standards representing 5, 10 and 20 mg *p* aminosalicylic acid/100 ml blood. 0.25, 0.50 and 1.0 ml of solution (1) and 15 ml 20% (w/v) trichloroacetic acid are diluted to 100 ml with water. These are referred to as '5, 10 and 20 mg solutions'. With standard solutions containing *p* aminosalicylate, 20 ml samples are treated as supernatant liquids of the test solutions, using *m* aminophenol standards, heating may be omitted.

Method A

Oxalated or freshly drawn capillary blood (0.2 ml) is added to water (3.2 ml), and the tube is allowed to stand for 30 min. Trichloroacetic acid (0.6 ml) is added, and the mixture is shaken and centrifuged. Supernatant liquid (2.0 ml), transferred to a centrifuge tube graduated at 4 ml, is heated with H_2SO_4 (0.5 ml) in a boiling water bath for 1 hr. The solution is cooled in tap water (approx. 15°), partially neutralized with NaOH (1.0 ml) and again cooled. Water is added to adjust the volume to 4.0 ml.

NaNO_2 solution (0.2 ml) is added, and the solution is shaken. After 5 min ammonium sulphamate solution is added, followed 20 \pm 1 sec later by naphthylethylenediamine (1.0 ml), the tube being shaken after each addition.

The pink colour is allowed to develop for at least 2.5 hr and is then compared with the appropriate standard solution. An Evans Electro Selenium Ltd portable photoelectric colorimeter with an Ilford 625 filter gives readings proportional to concentration if set to zero against a full reagent blank (2 ml 3% w/v trichloroacetic acid taking the place of the supernatant liquid). An Ilford 404 green filter though not giving fully linear results has also been found satisfactory if a calibration curve is prepared using all three standards (the 5, 10 and 20 mg solutions).

Method B

Method *A* is carried out to the coupling stage. 20 \pm 1 sec. after coupling with naphthylethylenediamine solution,

ethanol (1.0 ml) is added, the tubes are shaken, and the colours compared after 1 hr. These are proportional to concentration when using an Ilford 625 filter and a modified reagent blank (2.0 ml water taking the place of the supernatant liquid). The Ilford 404 filter is unsuitable for this method.

RESULTS

Decarboxylation and hydrolysis Our first experiments showed that solutions of *p* aminosalicylate heated with concentrated hydrochloric acid gave deeper colours than solutions treated with hydrochloric acid in the cold or heated with trichloroacetic acid. Sulphuric acid was substituted for hydrochloric to prevent the formation of hydroxyazo compounds during the diazotization stage. The strength of acid and the heating time finally adopted give complete decarboxylation, as is shown by carrying out the method on *p* aminosalicylate and *m* aminophenol solutions with and without heating (Table 1). Determinations carried out on 20 mg

Table 1 The conversion of *p* aminosalicylate into *m* aminophenol

(20 mg standards used in each case)

Exp no	Compound and treatment	Final concentration of <i>m</i> aminophenol expressed as	
		Colorimeter reading (100 \times \bar{E})	Percentage of result in Exp 1
1	<i>m</i> Aminophenol, not heated	54	100
2	<i>m</i> -Aminophenol, heated	55.5	103
3	<i>p</i> Aminosalicylate, not heated	17	31.5
4	<i>p</i> Aminosalicylate, heated	53	98

solutions of *m* aminophenol and *p* acetamidosalicylic acid show that the *N* acetyl derivative of *p* aminosalicylic acid is completely hydrolysed by this treatment. Thus the present estimation is one of total *p* aminosalicylate. When heating is omitted, solutions of the *N* acetyl compound give no colour reaction.

Diazotization Partial neutralization with sodium hydroxide gives the moderately acid medium usual for diazo coupling. Observations which led to this step being introduced are summarized in Table 2. Diazotization for 5 min was chosen as a convenient time, since the colour intensity/diazotization time curve showed only a very gradual decrease between 3 and 10 min. (Colorimeter reading at 3 min., 60, 5 min., 59.5, 10 min., 59, 15 min., 57). Minor changes in the temperature of diazotization introduce appreciable errors, all test and standard solutions should be placed in the same cooling bath.

Table 2 *Effect of pH on colour intensity in the estimation of p aminosalicylate as m aminophenol*

(20 mg standards used in each case)

Exp no	Decarboxyl ation medium	Diazotizing and coupling medium	Colour intensity (percentage of value in Exp 2)
1	Strongly acid (21.5N H ₂ SO ₄)	Strongly acid (unchanged)	51.5
2	Strongly acid (21.5N H ₂ SO ₄)	Less acid (partly neutralized with 8.1N NaOH)	100
3	Less acid (9N H ₂ SO ₄)	Less acid (unchanged)	81
4	Less acid (9N H ₂ SO ₄)	Strongly acid (9N H ₂ SO ₄ added until pH approx that of Exp 1)	12

Newhouse & Klyne (1949) allowed 10 sec between adding ammonium sulphamate and coupling. Our sulphamate time of 20 sec was adopted directly from their method. It gives colours about 20% deeper than we had formerly obtained using longer times (cf Table 3).

Table 3 *The effect of varying diazotizing and sulphamate times on the colour intensities obtained from p aminosalicylate and m aminophenol solutions*

(20 mg standards used in each case)

Exp no	Compound	Diazo tizing time (min)	Sulphamate time	Colori meter reading (100 × E)
1	p Aminosalicylate	5	20 sec	57
2	"	5	30 min	38
3	"	20	20 sec	49
4	"	20	10 min	44.5
5	m Aminophenol	5	20 sec	58
6	"	5	20 min	50
7	"	20	20 sec	53
8	"	20	20 min	43

Properties of the dye solutions The colours given by methods A and B follow Beer's law between 2.5 and 20 mg/100 ml levels using an Ilford 625 filter. The similar development (Fig 1) of the colours obtained from equimolar solutions of p aminosalicylate after heating and of m aminophenol confirms the identity of the two dyes. Unheated solutions of p aminosalicylate develop colour more rapidly than either of these two solutions. This indicates a structural difference between the dyes rather than a partial decarboxylation of the acid in the cold which would then give less of the same dye.

Both conclusions are borne out by the absorption spectra of the dyes (Fig 2). These were recorded by

Dr I D P Wootton, using a Beckmann spectrophotometer. The solutions used were those obtained by carrying through 20 mg solutions of p aminosalicylate and m aminophenol. The heated p aminosalicylate and the unheated m aminophenol solutions have identical spectra. The unheated p aminosalicylate solution gives a dye with the expected lower extinction values and an absorption maximum at a somewhat shorter wavelength.

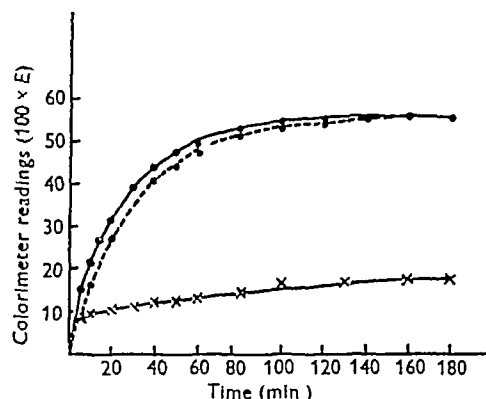


Fig 1 Rate of colour development in the estimation of p aminosalicylate and m aminophenol (20 mg standards, using method A) —•—, m aminophenol, unheated, —, p aminosalicylate, heated, —x—x—x, p aminosalicylate, unheated

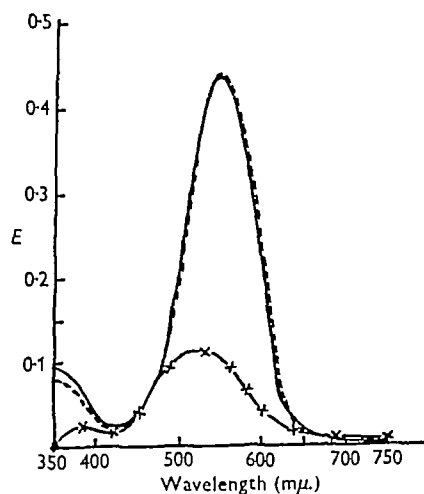


Fig 2 Absorption spectra of dyes (20 mg standards, using method A) —•—, m aminophenol, unheated, —, p aminosalicylate, heated, —x—x—x, p aminosalicylate, unheated

The colours produced in method A are stable to light and are unchanged after 90 hr at room temperature. The solution of the dye obtained from unheated p aminosalicylate is stable only for a few hours. The dye slowly separates as a flocculent precipitate, leaving a colourless solution. On shaking

the mixture the precipitate is dispersed, the solution appears clear and has the same colour intensity as it had before flocculation occurred. The precipitate can be separated easily by filtration. Since in the aqueous acid medium containing a high salt concentration the more acid dye produced from the unheated *p* aminosalicylate would probably be less soluble than its *m* aminophenol analogue, the precipitate was filtered off and the filtrate kept for some time. No further colour developed, thus the low colour intensity of this solution is not due to a solubility effect leading to incomplete dye formation.

Table 4 Recoveries of *p* aminosalicylate added to blood

(Aqueous *p* aminosalicylate solution (100 mg/100 ml) added to oxalated blood)

Blood <i>p</i> amino salicylate level (mg/100 ml)	No of exp	Recovery (%)	
		Range	Mean
10	17	89-102	96
20	8	91-102	98

Attempts were also made to follow the diazotization of *p* aminosalicylic acid and *m* aminophenol by means of free nitrous acid estimations. The *m* phenylenediamine method (see Welcher, 1947) used cannot be described as a strictly quantitative method, it does indicate, however, that the amount of nitrous acid used in the reaction is in excess of that required by a simple diazotization.

Recoveries The results of these are given in Table 4. The determinations were carried out on fresh oxalated blood to which the requisite amount of *p* aminosalicylate (stock standard solution) was added.

Penicillin did not interfere with the estimation. Sulphonamides and sulphones (Sulphetrone) do, of course, interfere. An unsuccessful attempt was made to overcome this difficulty by converting the diazotized *m* aminophenol to resorcinol and estimating the product by an adaptation of Selivanoff's reaction.

SUMMARY

1 A method for the estimation of 'total *p* amino salicylate' in capillary blood is described.

2 *p* Aminosalicylic acid and its *N* acetyl derivative are quantitatively converted to *m* aminophenol, this compound gives deeper colours than *p* aminosalicylate when diazotized and coupled with *N* 1 naphthylethylenediamine.

3 The main aspects of the method have been surveyed and the behaviour of *p* aminosalicylic acid and *m* aminophenol compared.

We would like to thank Dr L. A. Elson, Chester Beatty Research Institute, London, for valuable suggestions and criticism, Dr I. D. P. Wootton, Postgraduate Medical School of London, for determining the absorption spectra, and Mr D. E. Seymour of Messrs Herts Pharmaceuticals Ltd. for gifts of pure sodium *p* aminosalicylate, *p* acetamidosalicylic acid and *m* aminophenol.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 279th Meeting of the Biochemical Society was held in the Department of Biochemistry, School of Medicine, Leeds 2, on Friday, 23 September 1949, when the following papers were read

COMMUNICATIONS

The Effect of Thyroxine and Thiouracil on some of the Water-soluble Vitamins in Milk By R CHANDA, MARY L McNAUGHT and E C OWEN (*Hannah Dairy Research Institute, Kirkhill, Ayr*)

In a previous communication Chanda & Owen (1949) showed that thyroxine markedly affected the partition of P in cows' milk, and that the changes in the partition were correlated with simultaneous changes in the phosphatase content of the milk. They also showed that milk from cows treated with thiouracil exhibited changes of phosphatase content and P partition which were the reverse of

phosphate and orthophosphate being present (de Jong, 1941-2)

In the present experiment three pairs of cows were used. One pair acted as controls. A second pair received 10 mg thyroxine per day subcutaneously, while a third pair received 20 mg thiouracil subcutaneously. Composite 2 day samples of milk from each cow were analysed for 3 weeks before treat-

Table 1 *The riboflavin content and partition of vitamin B in milk*

Group of animals	Period	Phosphatase (arbitrary units)	(μg/100 ml. fat-free milk)				
			Total B ₁	Free B ₁	B ₁ phosphoric esters	Protein bound B ₁	Riboflavin
Control	1	53	42.9	21.1	16.8	3.15	92.9
	2	62	39.3	23.7	10.9	2.79	91.3
	3	83	38.8	27.6	6.4	2.59	89.6
Thyroxine	1	68	44.3	27.0	12.9	2.73	107.3
	2	28	43.5	12.9	25.4	3.80	96.3
	3	78	40.2	23.8	11.2	2.63	104.2
Thiouracil	1	38	48.1	21.2	21.1	3.57	115.9
	2	67	42.1	28.4	9.5	2.69	109.6
	3	66	41.0	27.4	8.5	2.77	103.3

those caused by thyroxine. In the same experiments thyroxine treatment caused the vitamin C in the milk to decrease by 26.3% and thiouracil caused it to increase by 22.2%, the average of pre-treatment figures for this vitamin in the milk of treated cows being 2.23 mg/100 ml. Houston, Kon & Thompson (1940) had found an inverse correlation between vitamin B₁ pyrophosphate and phosphatase in the milk of normal cows, and experiments were therefore planned to find whether thyroxine or thiouracil would alter the amount of B₁ phosphoric esters in the milk. The term 'B₁ phosphoric esters' is preferable to 'B₁ pyrophosphate', since the nature of the B₁-esters in milk of various species is still controversial, both pyro-

ment (period 1), for 3 weeks during treatment (period 2), and for 3 weeks after treatment (period 3). The average results for each period (Table 1) show that, like ester P in the previous experiment (Chanda & Owen, 1949), phosphoric esters of B₁ increased as phosphatase decreased when thyroxine was given, while when thiouracil was given the reverse occurred. The disturbance of the ratio of free to phosphorylated vitamin B₁ by thyroxine confirms the similar observation by Thompson (1945). Total riboflavin did not vary significantly during treatment, and, unfortunately, the partition of riboflavin into free and phosphorylated forms could not be investigated owing to lack of a method adaptable to routine analysis.

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Some Remarks on the Hydrolysis of Proteins By H. M. FLOWERS and W. S. REITH (REICH)

The Energy of Activation of the Tryptophanase-tryptophan Indole Reaction By FRANK C. HAPFOLD and G. A. MORRISON (*Department of Biochemistry, School of Medicine, University of Leeds*)

The energy of activation has been studied. The provisional figure of 18,200 cal/g mol is in the range of temperatures from 0 to 37° C given.

Absorption and Excretion of DL-Thyroxine Labelled with Radioactive Iodine By J. C. CLAYTON, AUDREY A. FREE, J. E. PAGE, G. F. SOMERS and E. A. WOOLLETT (*Research Division, Glaxo Laboratories Ltd, Greenford, Middlesex*)

DL-Thyroxine labelled with ^{131}I in the 3' and 5' positions has been prepared by an improved procedure (Borrows, Clayton & Hems, 1949), and its absorption and excretion by rats and cats have been studied.

Joliot, Courrier, Horeau & Sue (1944*a,b*), Joliot (1945) and Gross & Leblond (1947) have investigated the absorption and excretion of relatively large intravenous doses of labelled DL-thyroxine by rabbits and rats. In view of the doubts about the efficacy of orally administered thyroxine, we were particularly interested in its absorption from the digestive tract. We have also studied the behaviour of physiological doses by the intravenous and subcutaneous routes.

The labelled thyroxine solutions were shown to be biologically active (Smith, Emmens & Parkes, 1947) and all preparations were polarographically assayed for thyroxine (Borrows, Hems & Page, 1949). The urine, blood, bile, faeces and organs from the treated animals were digested and the radioactivity of an aliquot portion was measured in a jacketed Geiger Müller counter (Veall, 1948). The amounts of ^{131}I present in the bile, urine and faeces, as thyroxine, diiodotyrosine and iodide, were measured by Gross and Leblond's method (1947).

Experiments on rats that had received oral doses of 1.0 mg of labelled thyroxine per kg of body weight showed that thyroxine was absorbed from the gut, and that 95% of the dose was excreted during the first four days. The ^{131}I in the urine was present almost entirely as iodide, whereas that in the faeces was present in both organic and inorganic forms. Our results were in general agreement with those of Gross & Leblond (1947).

Anaesthetized cats were used to study the amount of ^{131}I in blood and its excretion in the bile and urine. The cats received single doses of 34–100 μg of labelled thyroxine per kg of body weight, subcutaneously, intravenously or by direct injection into the duodenal lumen. Absorption occurred from the intestinal lumen. For all routes, the amount of ^{131}I in the bile was related to the blood concentrations, the ^{131}I in the bile was present in approximately equal proportions in organic and inorganic forms. Appreciable amounts of ^{131}I were secreted into the stomach, but very little activity was found in the thyroid glands. About 6% of an intra duodenal dose was excreted in the urine during the first 6 hr after injection.

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Some Observations on the Co-enzyme of Phosphoglucumutase By L H STICKLAND

Trucco, Cardini, Paladini, Caputto & Leloir (1948) have recently shown that glucose 1 6 diphosphate (GDP) is the co enzyme of yeast phosphoglucumutase (PGM), and suggest that the claim of Kendal & Stickland (1938) that fructofuranose 1 6 diphosphate (FFDP) is the co enzyme was due to contamination of their FFDP with a trace of GDP. This communication aims at showing that, although Kendal and Stickland's FFDP was indeed contaminated with GDP, yet FFDP can take the place of the co enzyme of muscle PGM.

The evidence is

(1) The apparent affinity of yeast PGM is much smaller than that of muscle PGM for crude FFDP. According to Sutherland, Posternak & Cori (1949) the two enzymes have the same affinity for GDP.

(2) FFDP purified by repeated crystallization of the brucine salt is inactive to yeast PGM, but still active to muscle PGM. Roughly one half of the activity of the crude FFDP is due to its GDP content, which is of the order of 1 %.

(3) During acid hydrolysis of pure FFDP, co enzyme activity is lost *pari passu* with the hydrolysis of the first phosphoric acid. GDP, on the other hand, is completely inactivated by very mild acid hydrolysis (Trucco *et al* 1948).

(4) On alkaline hydrolysis of pure FFDP, re-

moval of 85 % of the phosphoric acid destroys the activity to muscle PGM, while similar treatment of the crude product leaves the activity to the yeast enzyme unchanged.

(5) Partial hydrolysis of the crude FFDP gives results consistent with the view that the activity is due one half to GDP and one half to FFDP.

FFDP obviously cannot participate in the chain of reactions suggested by Trucco *et al* as the mechanism of the action of GDP. It seems much more likely that FFDP acts by transferring a phosphoric acid to glucose 1 phosphate, to produce GDP, which is the true co enzyme. All the present experiments were done with a partially purified muscle PGM, the behaviour of a highly purified enzyme (Jagannathan & Murray Luck, 1949) is still to be studied.

Another point of difference between the enzymes from yeast and from muscle is that, whereas yeast PGM in the absence of GDP is completely inactive, partly purified muscle PGM is not. After a lag period the reaction begins slowly, gathers speed, and eventually reaches a rate equal to that found at optimal co enzyme concentration. This may possibly account for the fact that several workers have purified muscle PGM without observing that it needs a co-enzyme.

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Amino-acid Oxidase of *Neurospora crassa* By A E BENDER, H A KREBS (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield*) and N H HOROWITZ (*Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California*)

Horowitz (1944) reported that aqueous extracts of *Neurospora crassa* contained an amino acid oxidase capable of converting D α amino acids into the corresponding α ketonic acids and ammonia. The enzyme was found to be inactive against L amino acids, except L glutamic acid which was oxidized at a rate less than one fifth of that found for the D compound.

A strain of *N. crassa* obtained from the National Type Culture Collection (no 3411) was found by Bender & Krebs to produce a highly active amino acid oxidase, but only L amino acids were attacked by this enzyme. A second strain of *N. crassa*, which

was obtained from Mr G H Wiltshire, Cambridge, also produced an L amino acid oxidase.

The apparent discrepancy between these results and those reported by Horowitz were studied jointly at the Sheffield and Pasadena laboratories and was found to be due to strain differences. Under identical conditions some strains form a D amino acid oxidase only and some an L amino acid oxidase only. Other strains form both enzymes.

The formation of the two enzymes also depends on the composition of the medium. Both enzymes are formed (by suitable strains) when the synthetic medium of Horowitz & Beadle (1943) with 0.25 μ g

biotin per litre is used, but increasing the biotin concentration to $5 \mu\text{g/l}$ suppresses the production of the L enzyme by the Cambridge strain

The L-amino oxidase was present not only in extracts of the mycelium but also in the clear liquid culture medium. In some experiments over 80% of the total enzyme produced in the culture flask was present in the medium after 15–18 days of incubation. The D amino acid oxidase is not discharged into the medium.

Knight (1948) has recently described an L amino acid oxidase in acetone dried mycelia of several *Penicillium* species and of *Aspergillus niger*. This enzyme seems to differ in several ways from that found in *Neurospora crassa*. The quantity of L-enzyme produced by *N. crassa* can be of the order

of 100 times greater, on a weight for weight basis, than that produced by the other moulds. There are also differences in the relative rates at which different amino acids are attacked. The *Neurospora* enzyme, unlike Knight's, is not inhibited by narcotics such as octanol.

The L amino acid oxidase is the second oxidase found to be released by a mould into the medium, the first case being that of glucose oxidase (Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick 1945). Together with L amino oxidase, *N. crassa* releases a highly active catalase into the medium. Hence no accumulation of hydrogen peroxide is expected when the oxidase acts upon amino acids under normal growth conditions.

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Plasma and Tissue Protein Regeneration in Renal Disease with Hypoproteinaemia and Oedema

By FRED A. K. HERBERT (Section of Chemical Pathology, Department of Pathology, King's College, Newcastle upon Tyne)

A few studies hitherto reported show that in this type of renal disease the administration of a diet high in both protein and total calories causes positive N balance which may be maintained for many months, though restoration of plasma protein may be very slow (Peters, Bruckman, Eisenman, Hald & Wakeman, 1931; Liu & Chu, 1935; Keutmann & Bassett, 1935; Farr, 1938; Cowie, Jarvis & Cooperstock, 1930). The object of the present work was to determine whether cystine, given as a supplement to diets of a type expected to give the highest N balance so far obtainable, would increase the rate of plasma protein regeneration. Heavy

albuminuria involves loss of serum albumin, which is richer in cystine than any dietary protein. Some observations of Whipple and his collaborators suggested that cystine might have a specific effect on plasma protein regeneration (Madden, Noehren, Warach & Whipple, 1939; Robscheit Robbins, Miller & Whipple, 1943).

Four patients with nephritis, giving a history of insidious onset of oedema without gross haematuria, have been studied. Total serum protein ranged from 3.27 to 4.11%, albumin from 1.37 to 2.15%. Data were obtained for total N in diet, urine, and faeces, fluctuations in N/P/N of body fluid, serum

Case	1		2		3		4	
Sex	F		M		M		M	
Age	27		40		33		17	
Duration of illness	7 months		3 months		17 months		10 months	
Cystine given, g/day	0	0.5	0	2.7	0	2.7	0	2.4
Protein (g N/day)								
(a) Absorbed	7.56	7.57	17.33	17.28	17.29	17.39	16.93	17.17
(b) Catabolized	3.35	5.11	10.95	11.41	11.08	10.15	12.29	10.84
(c) Gained in tissues	2.23	0.87	3.89	2.74	3.38	4.48	1.32	3.34
(d) Gained or lost in plasma	+0.19	-0.07	0.00	-0.14	+0.27	-0.14	+0.36	+0.04
(e) Lost as urinary protein	1.79	1.66	2.17	2.79	2.20	2.47	2.29	2.13
Albumin			0.31	0.48	0.36	0.43	0.67	0.82
(f) Total protein synthesized (c + d + e)	4.21	2.46	6.37	5.87	6.21	7.24	4.64	6.33

proteins, plasma volume, and urinary protein. Total calorie intakes ranged from 2613 to 3412 per day. Protein intake ranged from 116 to 124 g per day except in case 1, in which there was a low urea clearance. Case 2 was complicated by recurrent infection, and very slight pyrexia was present during the balance study. The diet was given for 4-9 days in preparation, for the next 7-10 days as a balance period, and for a further 7-10 days' balance period with added cystine. The dose of cystine was planned to bring the cystine lysine ratio of the dietary protein up to that of serum

albumin. The essential findings are given in the table.

On diet alone, there were high positive N balances due to tissue protein regeneration. A high rate of serum protein regeneration was offset by albuminuria. In no case did the cystine improve the rate of serum protein regeneration appreciably. In case 3, cystine appeared to produce a definite improvement in tissue protein regeneration. In case 4, a growing boy, a moderate rate of tissue protein regeneration on diet alone was greatly increased by the cystine supplement.

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Transport of Radioactive Na⁺ and K⁺ Through Gastric Mucosa By E. EVA CRANE and R. E. DAVIES (*Unit for Research in Cell Metabolism (Medical Research Council), Department of Biochemistry, University of Sheffield*)

Experiments with radioactive ions to discover the flux of Na⁺ and K⁺ across gastric mucosa under a variety of conditions have been carried out as part of an investigation into the secretory and electrical activity of this membrane. Isolated frog gastric mucosa was mounted in the apparatus described by Crane, Davies & Longmuir (1948). The nutrient solution was frog bicarbonate saline, the secretory solution was either 0.12 M NaCl or 0.12 M KCl. The solution bathing one side contained a known concentration of ²⁴Na or ⁴²K, the solution bathing the other side was changed periodically and its radioactivity measured with a liquid counter.

A complication arose when it was discovered that even when the radioactive isotope (in a solution of total molarity 0.12) was diluted 250 fold with water, losses of activity up to 30% occurred due to exchange of radioactive ions with ions in the walls of glass vessels, syringes, etc. The activity could be recovered by washing with solutions of the stable isotope, and the loss of activity did not occur when dilutions were made with 0.12 M NaCl or 0.12 M KCl.

For any one resting mucosa the rate of transport of Na⁺ or K⁺ remained constant to $\pm 15\%$, but increased rapidly if the mucosa was mechanically damaged as indicated by the fall of the potential difference across it. Experimental results given in Table 1 are based on averages for at least three consecutive half hour periods. They show that the

rates of transport of Na⁺ and K⁺ from the secretory to the nutrient side were almost the same. In the opposite direction the rate of transport of Na⁺ was

Table 1 *Rates of transport of Na⁺ and K⁺ across resting isolated frog gastric mucosa*

Direction of transport	Secretory to nutrient side		Nutrient to secretory side	
	Na ⁺	K ⁺	Na ⁺	K ⁺
Ion carrying activity	Na ⁺	K ⁺	Na ⁺	K ⁺
Concentration (mm)	120	120	110	4.5
No mucosae studied	5	7	4	6
Rate of transport (μ /cm ²)				
Mean	22	21	29	1.5
Range	10-39	10-35	20-39	0.8-2.6

$$1 \mu = 6.28 \times 10^4 \text{ univalent ions/sec} \\ = 1.04 \times 10^{-8} \text{ mm univalent ion/sec}$$

similar, the rate of transport of K⁺ (whose concentration in the nutrient solution was only 4.1% of the Na⁺ concentration) was 5% of the rate of Na⁺ transport. Thus there was no appreciable directional asymmetry of the type observed in frog skin for which the influx was about ten times the outflux (Ussing, 1949).

In nine experiments with ⁴²K, the rate of transport of K⁺ in both directions was reversibly increased up to sevenfold when histamine was added to the nutrient solution. This increase was not

dependent upon the initiation of acid secretion. A comparable effect of adrenaline on the rate of transport of Na^+ through frog skin was observed by Barker Jørgensen (1947) and Ussing (1949).

Experiments in which an applied electric current was passed through the mucosa in either direction showed that K^+ and/or Na^+ can carry at least part

of the current (cf Crane, Davies & Longmuir, 1948, Davies & Ogston, 1949). It is of interest that the electric currents which can be maintained in an external circuit by isolated frog gastric mucosa (Crane *et al* 1948) are of the same order as the cation currents which continuously cross the mucosa (Table 1).

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Observations on the Organic Matrix of Bone *School and Hospital, Leeds 1)*

By H. J. ROGERS (*Biological Research Unit, Dental*

During the normal resorption of bone and in some pathological conditions such as hyperparathyroidism the organic matrix as well as the inorganic salts of the bone are removed (e.g. Burns & Henderson, 1946). Thus any understanding of the process of resorption must take into account the removal of the organic as well as the inorganic material. Moreover, such is the paucity of our knowledge of the biochemical mechanisms underlying the resorptive process that either the removal of the hydroxyapatite or of the integrating organic matrix may be the primary step. The present work is concerned with some of the constituents of the organic matrix of bone, their quantitative changes with age and their distribution in the bone.

The amount of total N, calcium, and phosphorus in ether extracted dried samples of human bone powder has been measured. The amount of reducing substances and hexosamine has also been measured after acid hydrolysis of the powder. The samples studied were thoroughly cleaned pieces of femur taken from the centre of the shaft of bones obtained from people dying as the result of accidents. The results obtained show that the concentration of total nitrogen falls with age, a result anticipated by

Roche & Garcia (1936) for rats and more recently by Strobino & Farr (1949) for oxen.

The amount of reducing substances (3.0–4.5% expressed in terms of glucose on dry bone powder) and of hexosamine (0.10–0.26% expressed as glucosamine HCl on dry bone powder) also show a considerable reduction. The fall in concentration of both protein and 'polysaccharides' is greatest in the early years of life (12 months–14 years).

The nature of the polysaccharides and other 'reducing substances' present in bone is under investigation. Levene (1925) reported, without giving details, the isolation of a sulphated polysaccharide from bone, and Pincus (1948) claims the isolation of chondroitin sulphate from dentin. It has been found that during the process of decalcifying bone powder by cold 2N-HCl (3 hr treatment at 25°C) about 30% of the N and of 'polysaccharide' is rendered soluble.

Ground sections of bone have been stained by a modification of the Hotchkiss (1947) procedure for polysaccharides and high concentrations of staining material have been found in the Haversian canals and very low concentrations distributed in the matrix. Decalcified sections have yielded similar results.

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Preliminary Observations on the Metabolism of the Alveolar Bone of Mammals By
S WEIDMANN and H J ROGERS (*Biological Research Unit, Dental School and Hospital, Leeds 1*)

One of the most serious features of many types of paradontal disease (pyorrhea) is the loss of part of the alveolar bone which normally encases the roots of the teeth and thus holds them firmly in position. The bone may either be resorbed locally around one or more teeth, a condition which is often associated with deep bacterially infected pockets between the 'gum' and tooth, or, sometimes, a general horizontal resorption of the alveolus may appear to take place. A knowledge of the metabolism of this bone taken in comparison with that of members of the rest of the skeleton might, therefore, be of considerable value in helping forward our understanding of the general syndrome of pyorrhea.

The rate of exchange of radioactive phosphorus (^{32}P) has been investigated using cats. These animals were chosen for the preliminary work because the relative degree of calcification of the alveolus, cleaned by a flotation technique, was similar to that found for *Homo sapiens*. Cats suffer with a mild paradontal condition, but it was quite possible to select radiographically 'normal' animals, i.e. those which showed no apparent resorption of the alveolar bone on X ray examination. A solution of Na_2HPO_4 containing marked phosphorus was injected subcutaneously and the cats killed by exsanguination under anaesthesia, after varying periods of time. The level of the radioactive element in the alveolar

femur shafts and heads and in the blood serum was compared with the total concentration of phosphorus ($^{31}\text{P} + ^{32}\text{P}$) in the samples. In some experiments the level of radioactive phosphorus in the plasma was maintained approximately constant during the experimental period by repeated daily injections of the labelled salt solution, urine measurements were used to reflect the plasma level in such experiments.

The rate at which the phosphorus enters the bone salt of the trabecular alveolar bone is very much less than the corresponding rate for the trabecular bone of the epiphyses and little greater than that for the shaft of the long bone.

It is difficult to interpret this result by the hypothesis, so far unproved, that the exchange of phosphorus in the bones of the living animal is entirely due to the ionic exchange which takes place in powdered bone ash, and is thus a function of the degree of contact between the hydroxyapatite crystals and the circulating plasma (Neuman & Riley, 1940). If this were the whole explanation we should have to assume that the surface of crystals exposed in the trabeculae of the alveolar bone is only slightly different from that in hard bone, a conclusion difficult to accept in view of the histological and radiographic appearance of the bone.

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The 'Early-lag' Phase of *Aerobacter aerogenes* By S DAGLEY, E A DAWES and G A MORRISON
(*Department of Biochemistry, School of Medicine, Leeds*)

Working with an aerated synthetic glucose ammonium medium, Lodge & Hinshelwood (1943) found that bacteria taken early in their logarithmic growth phase showed 'early lag' when inoculated into a fresh lot of medium of the same composition. Several hours after the end of the logarithmic phase, 'late lag' developed, whilst at some intermediate point there was a minimum in the curve relating lag and 'age'. We have shown that these relationships are greatly influenced by conditions both prior to and during growth in the basal medium. In the work summarized below, bacteria from a stock maintained either on slopes or in broth were subcultured three times in succession in the basal medium before inoculation into the medium in which growth was followed.

(1) Cells from a stock kept on slopes showed no

early lag at ages for which the original workers (1943) demonstrated considerable lag, their strain being maintained by serial subculturing in broth. Slope maintained cells, however, showed lag when harvested and washed.

(2) The same strain when kept for 6 weeks in broth showed marked early lag in unaerated media, falling sharply to zero about one third of the way through the logarithmic phase. Beyond this point, i.e. whilst rapid cell division was still in progress, a later lag phase developed. Although, therefore, the position of the minimum in the lag age curve has formerly been regarded as approximately coincident with the onset of the stationary phase, work with the present strain indicates that the two phenomena are not necessarily connected.

(3) Cells grown in unaerated media showed a few hours' lag at all ages when transferred to the synthetic medium through which air was bubbled, but this lag was practically abolished by three successive subcultures in the presence of the air stream. Cells which had been stored in broth, as in (2), and then trained in this way to optimum aerobic growth were used as inocula for both aerated and unaerated media. In both cases, although minimum lag was given towards the end of log

arithmic growth, the lag-age curves were much shallower than in (2), where inocula were taken from unaerated cultures.

(4) Viable counts of cultures with early lag showed that in this phase cell division is not absent, but that there is a definite logarithmic rate of growth one third to one quarter the normal value. The latter is the rate which is measured by turbidimetric methods or by haemocytometer counts.

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Influence of Amino-acids, and Compounds in the Krebs Oxidation Cycle, on 'Early Lag'

By S. DAGLEY, E. A. DAWES and G. A. MORRISON (*Department of Biochemistry, School of Medicine, Leeds*)

Lodge & Hinshelwood (1943) obtained direct experimental evidence for the view that early lag in a synthetic medium is due to diffusion from the cells, at high dilution, of the intermediates necessary for rapid cell division to commence. If this view is accepted it is seen that, by studying the growth responses evoked by the addition of small amounts of various compounds, it may be possible to gain information about the nature of these essential intermediates. The following results were obtained with cells trained to the basal medium by three serial subcultures.

(1) Using inocula from a slope maintained stock taken from the early logarithmic phase but showing no lag period (see Dagley, Dawes & Morrison, 1949), the addition to the basal medium of any of the following compounds separately, at concentrations of about 10 parts per million, raised the initial growth rate significantly above its normal value: methionine, glutamate, aspartate, α -ketoglutarate, oxalacetate, succinate. By the time that visible growth appeared, growth rates had in all cases reverted to normal, although faint turbidity was

reached much earlier when the additions were made.

(2) With the exception of aspartate, lag produced by washing cells from slope stocks was also partly removed by these additions.

(3) Malate and fumarate did not produce this effect over the same region of concentration; fumarate was definitely inhibitory and malate slightly so.

(4) Early lag is a phase of reduced growth rate (Dagley *et al.* 1949). For cells exhibiting this phase, plate counts showed that the various additions at the concentrations stated raised this low rate of growth to a value approaching normal.

It is seen that whilst several compounds (and related amino acids) taking part in the Krebs cycle produce growth responses, others do not. Any theory assigning to the Krebs cycle a place of importance in the economy of the bacterial cell must take this fact into account, as well as our observation that fluoracetate had little adverse effect on bacterial growth, iodoacetate, over the same concentration range, is a powerful inhibitor.

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Lodge, R. M. & Hinshelwood, C. N. (1943) *J. chem. Soc.* p. 213

Factors Influencing the Polysaccharide Content of *Escherichia coli* By S. DAGLEY and E. A. DAWES (*Department of Biochemistry, School of Medicine, Leeds*)

Cells grown on an amino acid medium of known composition were washed, treated with 2N H_2SO_4 for a length of time known to ensure complete hydrolysis of the polysaccharide, and the resulting

reducing sugar estimated by the Somogyi (1937) method. Expressing the results as mg polysaccharide per 100 mg dry bacterial weight a storage of about 7% was obtained in the absence

of sugar, but when glucose, lactose or galactose were added the storage rose with concentration to about 18%, with a sugar concentration of 2 g/l Dawson & Happold (1943) reported no storage with mannose and we find that additional storage with this sugar and with fructose is slight. Storage was studied when the various sugars were present at a pH range from 6 to 9. For glucose and galactose, which give good storage at pH 7.5, alkaline or acid conditions had a marked adverse effect on storage, which is probably due to the fact that under these conditions little sugar is consumed before the stationary phase is reached. Storage in the presence of mannose and fructose is not so dependent upon pH, the low value at adverse pH values being similar to that under favourable conditions.

In an attempt to elucidate the function of polysaccharide storage its value was determined in the various phases of growth of the bacteria both in the amino acid medium and in a basal glucose ammonium medium. During the stationary phase the storage was reduced by almost half during a

period of 40 hr. It might be suggested that in this phase the cells were utilizing stored polysaccharide when foodstuffs in the medium were no longer available, but the following observations do not support this view. The fall in storage is not exactly coincident with the cessation of cell division and the same storage is obtained in the basal ammonium medium whether exhaustion of glucose determines the onset of the stationary phase or whether glucose is in excess and division ceases because the nitrogen source is exhausted.

The phases of growth were followed in this work by turbidity determinations and we have repeatedly confirmed the observation of Monod (1942) that when cell division ceases there is a definite fall in bacterial mass during the course of several hours. Although this might be ascribed to consumption of cell material, such as polysaccharide, during the resting phase this is not substantiated by the fact that the turbidity falls as soon as the stationary phase is reached, whereas the polysaccharide storage does not.

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Experiments on the Mobility of the Methyl Group in Dimethylacetothetin, $(\text{CH}_3)_2\text{S}^+ \text{CH}_2\text{COO}^-$

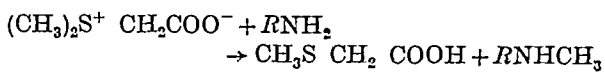
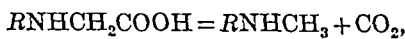
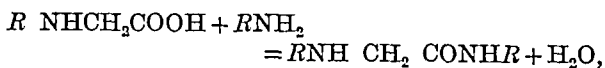
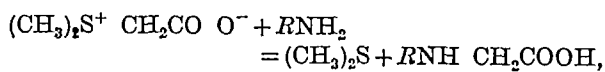
By F CHALLENGER and PATRICIA FOTHERGILL (Department of Chemistry, The University, Leeds)

Willstätter (1902) showed that at about 300° betaine, $(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COO}^-$, is partly converted to methyl dimethylamino acetate, and Challenger, Taylor & Taylor (1942) found that the mobile methyl group can be transferred by heat to compounds of S, Se and Te giving $(\text{CH}_3)_2\text{S}$, $(\text{CH}_3)_2\text{Se}$ and $(\text{CH}_3)_2\text{Te}$. With aromatic primary amines, RNH_2 , formation of RNHCH_3 occurs and disulphides, RS SR , also undergo methylative fission to RSCH_3 . After the isolation of dimethyl β prothetin chloride, $(\text{CH}_3)_2\text{S}^+(\text{Cl}^-)\text{CH}_2\text{CH}_2\text{COOH}$, from *Polysiphonia fastigiata* by one of us and Simpson (1948), Maw & du Vigneaud (1948) found that this thetin salt and also the chloride of dimethylacetothetin, $(\text{CH}_3)_2\text{S}^+\text{CH}_2\text{COO}^-$, supported growth of rats on a diet deficient in methyl donors but containing homocystine. The occurrence of biologically labile methyl in both these thetins was further demonstrated by Dubnoff & Borsook (1948), who showed that the chlorides could methylate homocystine to methionine using liver or kidney preparations of rats, guinea pigs and hogs.

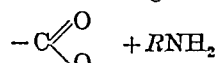
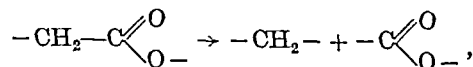
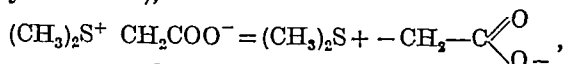
It was of interest to find whether the mobility of the methyl group in the thetins could be detected as in the case of betaine. We find that when the anhydride of dimethylacetothetin hydroxide, $(\text{CH}_3)_2\text{S}^+\text{CH}_2\text{COO}^-$, is heated with aniline, *p* toluidine or β naphthylamine the *N* methyl compounds of these bases, RNHCH_3 , are formed and characterized as the *N* nitroso-derivatives, RN(NO)CH_3 , or as the acetyl derivative. Much $(\text{CH}_3)_2\text{S}$ and CO_2 are evolved and considerable quantities of the diarylurea, CO(NHR)_2 , are formed. Anilinoacetanilide, $\text{C}_6\text{H}_5\text{NHC(O)CH}_2\text{NHC}_6\text{H}_5$, was also isolated, a type of compound repeatedly observed in the betaine experiments, where however no diarylurea was detected. The methylations effected by betaine and thetin may involve a positive or a neutral methyl radical (Challenger, 1945, 1947). It is conceivable that the methylation of the amines may be effected by a neutral $-\text{CH}_2-$ radical or even by the decarboxylation of an arylglycine. The diarylureas may perhaps arise from decomposition of a more complex compound or from the free radical

$-C-\ddot{O}-$ (see below) Further work is in progress

Possible reactions (compounds underlined have been detected)



(transmethylation, no derivative of CH_3SCH_2COOH yet isolated),



Gaseous CO_2 and boiling aniline give no diphenyl urea. The interconversion of the thetin and the isomeric ester, $CH_3SCH_2COOCH_3$, has not yet been effected

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Carbohydrates Present in the Subterranean Organs of some Members of the Compositae

By J S D BACON and J EDELMAN (*Department of Biochemistry, The University, Sheffield*)

Extracts made by disintegrating Jerusalem artichoke tubers with water in the Waring Blendor contained about 2 mg/ml of reducing substances. About 100 mg/ml of reducing sugar (estimated by the method of Miller & Van Slyke, 1936) was liberated on hydrolysis with 0.5% oxalic acid at 100° for 30 min. Of this four fifths was fructose (see Bacon & Bell, 1948) while the remainder was removed by the action of glucose oxidase (Keilin & Hartree, 1948) and was therefore glucose. Increasing concentrations of ethanol precipitated increasing amounts of polysaccharide material, but all fractions so obtained were soluble in cold water (cf Thaysen, Bates & Green, 1929).

Examination of such extracts by the method of paper partition chromatography (Partridge, 1948) showed the presence of small amounts of free fructose and of a series of components containing fructose with R_F values (in three solvent mixtures) ranging from those for sucrose to zero. In chromatograms run for 2-5 days with butanol acetic/water mixture at least six such components could be seen in addition to an undifferentiated zone of R_F below 0.02.

A similar series of components was found in extracts of the subterranean organs of five other species of Compositae.

One of the components has been tentatively identified as sucrose by

- (1) The R_F values in three solvents
- (2) The lack of reducing power as judged by the use of $AgNO_3$ and benzidine sprays
- (3) Its disappearance from chromatograms of tuber extracts treated with an invertase preparation from yeast
- (4) Extraction from the paper and estimation of fructose and total reducing sugar after mild acid hydrolysis
- (5) Hydrolysis by invertase *in situ* on a two dimensional chromatogram and subsequent identification of glucose and fructose

Similar methods have been applied to the examination of the other components of the extract. Additional information as to their composition has been obtained by the use of the enzyme preparation described in the following abstract.

- Bacon, J S D & Bell, D J (1948) *Biochem J* 42, 237
 Keilin D & Hartree, E F (1948) *Biochem J* 42, 221
 Miller B F & Van Slyke, D D (1936) *J biol Chem* 114, 583
 Partridge, S M (1948) *Biochem J* 42, 238
 Thaysen, A C, Bates, W E & Green, B M (1929) *Biochem J* 23, 444

REFERENCES

The Action of an Enzyme Preparation from *Helianthus tuberosus* L. (Jerusalem artichoke) on Carbohydrates Present in the Tubers By J EDELMAN and J S D BACON (*Department of Biochemistry, The University, Sheffield*)

Green (1887) described an enzyme hydrolysing inulin in extracts of tubers of the Jerusalem artichoke, which he named inulin-ferment. Other authors have studied inulases from plants, e.g. *Cichorium Intybus* L. (chicory) (Wolff & Geslin, 1918) and from moulds (see Oppenheimer & Pincussen, 1929).

Extracts of the tubers made as described in the previous abstract showed on incubation a marked negative change in optical rotatory power which could be correlated with an increase in reducing substances. The quantitative relations were consistent with the hypothesis that fructose was being liberated from material having a negative rotation. No such change was observed in boiled extract. The activity was greatest at about pH 5.

Attempts were made to concentrate and purify the enzyme activity. The standard substrate solu-

tion used was a boiled extract of tubers, adjusted to pH 5.0 with acetate buffer and filtered. The most active preparation obtained liberated about 9 mg of reducing sugar when 0.5 ml was added to 2.5 ml of standard substrate solution and incubated for 30 min at 40°. This represents a 20 fold concentration.

Paper partition chromatography (Partridge, 1948) has been used to separate the components of the standard substrate solution. The action of the enzyme preparation upon these and upon inulin was investigated. Both glucose and fructose are among the products of hydrolysis and intermediates have been detected resembling in R_f value the components of the freshly extracted juice (see preceding abstract). The rate of sucrose hydrolysis was only about one twentieth of that of the standard substrate under the test conditions.

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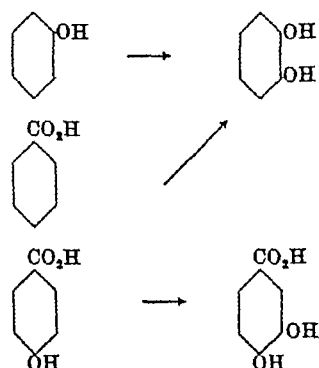
Oppenheimer, C. & Pincussen, L. (1929) *Die Methodik der Fermente* 912. Leipzig: Thieme.

Partridge, S. M. (1948) *Biochem. J.* **42**, 238.

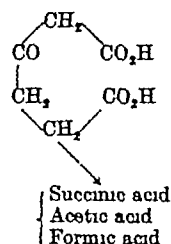
Wolff, J. & Geslin, B. (1918) *Ann. Inst. Pasteur*, **32**, 71.

The Mechanism of the Bacterial Oxidation of Certain Aromatic Compounds, together with the Preparation and Properties of a Cell-free Enzyme System which Accomplishes Ring Cleavage By W. H. PARR, R. A. EVANS and W. C. EVANS (*Biochemical Laboratories, University College of Wales, Aberystwyth*)

It has been established that vibrio O1 (Happold & Key, 1932) oxidizes the following aromatic compounds through the following intermediates (Evans & Happold, 1939; Evans, 1947; Kilby, 1948; Evans, Parr & Evans, 1949):

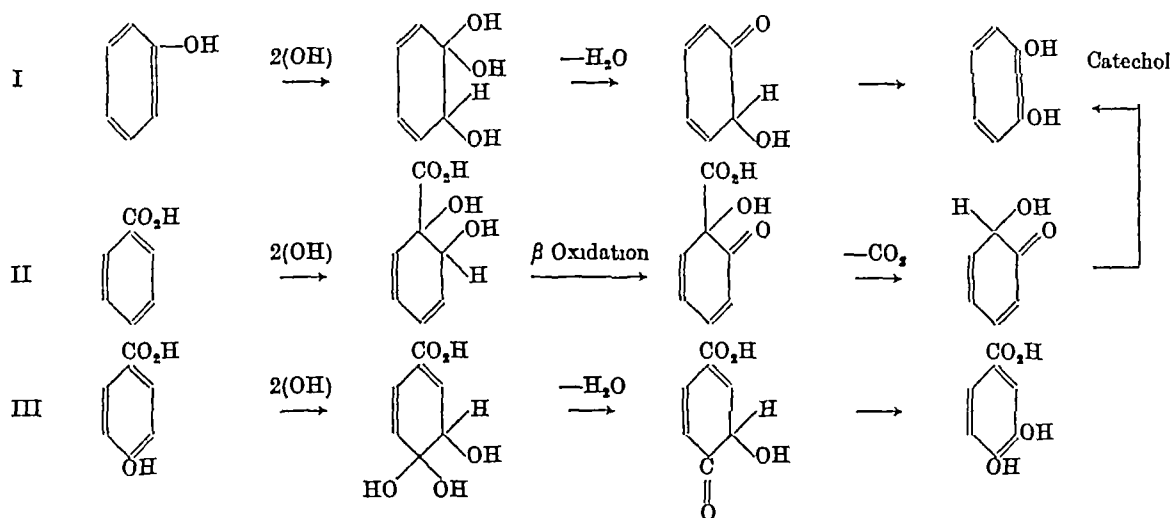


By the application of the simultaneous adaptation technique, Stanier, Sleeper, Tsuchida & MacDonald (1949) have concluded that *Pseudomonas fluorescens*, capable of metabolizing these substrates, does so in a similar manner. This we have confirmed,



by chemical isolation of intermediates, working with their organisms. An important point, brought out by the Stanier technique, is the essentially distinct pathways of benzoic and *p* hydroxy benzoic acids, during the initial stages of their metabolism (Stanier *et al* 1949)

To account for the intermediates isolated, we suggest the following mechanisms for the oxidations—they are visualized as occurring by the aid of H_2O_2 (or OH free radicals)



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 Happold, F. C. & Key, A. (1932) *J Hyg, Camb*, 32, 573
 Kilby, B. A. (1948) *Biochem J* 43, v
 Stanier, R. Y., Sleeper, B. P., Tsuchida, M. & Macdonald, D. L. (1949) Private communication, and *Nature, Lond* (in the Press)

Some Observations on the so-called 'Bracken Poisoning' of Farm Animals, and the Aneurin Inactivating Mechanism of Various Plants By H. E. ROBERTS, E. T. R. EVANS and W. C. EVANS (*University College of Wales, Aberystwyth*)

We have already confirmed the presence of a mechanism in the common bracken (*Pteris aquilina*) (Evans, E. T. R. & Evans, W. C. 1949, Evans, W. C. & Evans, E. T. R. 1949) which renders aneurin in the diet unavailable to the animal. These experiments, together with others (Weswig, Freed & Haag, 1946, Thomas & Walker, 1949), were done on rats. We now report the experimental production of 'bracken staggers' in the

horse, together with observations on the condition. The aneurin and pyruvic acid blood levels of such an animal presents a typical picture of avitaminosis B₁. Daily injections of 50–100 mg of aneurin, subcutaneously, without changing the bracken diet led to a rapid recovery of the animal.

Some properties of the aneurin inactivating mechanism were discussed.

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 Thomas, B. & Walker, H. F. (1949) *J Soc chem Ind, Lond*, 68, 6
 Weswig, P. H., Freed, Anna M. & Haag, J. R. (1946) *J biol. Chem* 165, 737

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

The potato eelworm hatching factor

- 1 The preparation of concentrates of the hatching factor and a method of bioassay By C T CALAM, H RAISTRICK and A. R. TODD
- 2 Purification of the factor by alkaloid salt fractionation Anhydrotetrone acid as an artificial hatching agent By C T CALAM, A. R. TODD and W S WARING
- 3 Concentration of the factor by chromatography Observations on the nature of eclepic acid By D H MARRIAN, P B RUSSELL, A. R. TODD and W S WARING
- 4 *Solanum nigrum* as a source of the potato eelworm hatching factor By P B RUSSELL, A. R. TODD and W S WARING
- 5 Attempts to prepare artificial hatching agents Part I Some furan derivatives By P B RUSSELL, A. R. TODD and W S WARING
- 6 Attempts to prepare artificial hatching agents Part II Some active arylidene Δ^8 γ butenolides and related compounds By D H MARRIAN, P B RUSSELL and A. R. TODD

The fermentation process in tea manufacture 10 The condensation of catechins and its relation to the chemical changes in fermentation By E A H ROBERTS

The deamination of glycine by α radiation from the disintegration of boron in a nuclear reactor By W M. DALE, J V DAVIES and C W GILBERT

Observations on antisera against complexes of di 2 chloroethyl sulphide and protein. By D S FLEMING, A M. MOORE and G C BUTLER

Investigations into cholinesterase levels in serum and cerebrospinal fluid of psychotic patients By D F EARLY, R E HEMPHILL, M. REISS and E BRUMMEL

Amino acid decarboxylases of rat liver By G H SLOANE-STANLEY

Methods for the determination of *N* methyl 2 pyridone 5 carboxylamide and of *N* methyl 2 pyridone-3-carboxylamide in human urine By W I M. HOLMAN and D J DE LANGE

The terminal peptides of insulin By F SANGER

Regulation of urinary steroid excretion 2 Spontaneous changes in the pattern of daily excretion in mental patients By M. REISS, R E HEMPHILL, J J GORDON and E R COOK

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The use of buffered columns in the chromatographic separation of 2 4-dinitrophenyl amino acids By S BLACKBURN

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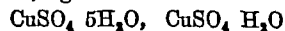
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stances and CHCl_3 , CCl_4 and CS_2 , formulae may be used in the text as abbreviations, particularly in the experimental portion, at the discretion of the editors. With salts it must be stated whether or not the anhydrous material is used, e.g. anhydrous CuSO_4 , or which of the different crystalline forms is indicated, e.g.



Descriptions of Solutions Solutions of common acids and bases should always be expressed in terms of normality (N), and salts preferably in terms of molarity (M), e.g. N-HCl, 0.1M NaH_2PO_4 . Fractional concentrations should preferably be expressed in the decimal system, e.g. 0.25N HCl (not N/4 HCl). The term '%' must be used in its correct sense, i.e. g/100 g of solution. [For 'per cent by volume', i.e. ml./100 ml., the term '% (v/v)' may be employed. To indicate that a given weight of substance is contained in 100 ml. of solution, the term '% (w/v)' (weight per volume) may be used.]

Symbols and Abbreviations These are given on pp. 3-7 of these notes. Spectrophotometric terms and symbols are those proposed by the Society of Public Analysts and other Analytical Chemists (see *Analyst*, 1942, 67, 164). The attention of authors is particularly drawn to the following symbols: $m = (\text{milli}) = 10^{-3}$ and $\mu = (\text{micro}) = 10^{-6}$. Note also that ml. (millilitres) should be employed instead of c.c., and μg (micrograms) instead of γ .

Nomenclature of Micro-organisms Binominal Latin names of micro-organisms, the generic name only with a capital, must be used in accordance with the International Rules of Nomenclature. Binominals should be underlined once (for *italic*) in the typescript. A name must be given in full at the first mention in a paper, in subsequent mention the generic name may be abbreviated, but the abbreviation must be unambiguous. Single initial letter abbreviations should, in general, be avoided (thus *Staph aureus*, *Strep pyogenes* not *S aureus*, *S pyogenes*). Scientific epithets or trivial names are not underlined and should be without capitals.

Microfungi should be designated as in Ainsworth & Bisby's *A Dictionary of the Fungi* (1945, 2nd ed., Kew Imperial Mycological Institute).

Bacteria The Editorial Board prefers that the nomenclature of Bergey's *Manual of Determinative Bacteriology* (1948, 6th ed., London: Bailière, Tindall and Cox) should be followed. Where authors wish, for good reasons, to use a name other than that in Bergey's *Manual*, the name as in Bergey's *Manual* should be inserted in brackets at the first full citation, thus *Chromobacterium prodigiosum* (*Serratia marcescens*).

Reprints Where at least one author of a paper is a member of the Biochemical Society, twenty-five reprints are supplied free of cost. If the supply of paper permits, an author may purchase additional reprints if he notifies the Press on the appropriate form immediately the proof of the paper is received, but only in exceptional circumstances will more than a total of 175 additional reprints be supplied.

ABBREVIATIONS AND SYMBOLS

General

The following list gives abbreviations which may be used in the text of papers, as distinct from symbols which are essentially for use in mathematical formulae but may also be used in the text

In the Introduction, Results, Discussion and Summary sections of papers the use of abbreviations should be very sparing. Thus, abbreviations for units are to be used, but not contractions of non-technical words or chemical symbols as abbreviations for elements, groups or compounds, unless this use is justified by special circumstances

The abbreviation for the plural is the same as that for the singular, unless confusion is likely to arise. Thus 'centimetres' is 'cm' not 'cms'

The symbols M, k, d, c, m, μ , m μ and $\mu\mu$ for multiples and sub-multiples may be prefixed to the symbol for any unit

Chemical Formulae and Abbreviations

Elements, groups, inorganic compounds (except water), and chloroform, carbon tetrachloride and carbon disulphide are to be denoted by symbols in the Experimental portion of the paper, but not generally in other portions. Diatomic molecules are to be denoted appropriately, e.g. H_2 , O_2 , N_2 , I_2

The abbreviations marked † for certain chemical compounds may be used, but the full name (with the abbreviation in parentheses) must be given the first time the compound is mentioned in each paper. Among the abbreviations marked † is a set of abbreviations for the names of amino-acids and their residues taken from a review by Brand & Edsall (1947, *Ann. Rev. Biochem.* 16, 224). These abbreviations are to be used only to represent the structures of polypeptides (e.g. Gly Ala = glycylalanine), and the empirical formulae of proteins (cf. the example given by Brand & Edsall). A key to the abbreviations must be given at the first mention in each paper. The abbreviations GSH and GSSG are used for reduced and oxidized glutathione

In chemical names elements carrying substituents are shown by symbols in italics, e.g. *N*-methyl-, used, if necessary, with primes or superscript numerals, e.g. N^4 -acetyl-sulphanilamide. The following forms are used: C_{20} acid, an acid containing 20 C atoms, $C_{(3)}$ or C-3, the atom numbered 3, *C*-Me, carbon-methyl group

Isotopes The atomic weight is indicated by superscript figures *before* the symbol of the element, e.g. ^{14}C , ^{15}N

Symbols

In general the *Biochemical Journal* follows the 'Report of a Joint Committee of the Chemical Society, the Faraday Society and the Physical Society on Symbols for the Thermodynamical and Physico-Chemical Quantities and Conventions relating to their Use' (1937). The following list includes some of the more frequently used symbols, and certain special symbols not included in this report

Symbols for purely mathematical constants and operators are printed in roman type, e.g. *e*, base of natural logarithms. Other symbols for quantities, as distinct from

abbreviations, are printed in italic type, when they are not Greek, e g *d* (density), *E* (extinction or electrode potential), *k*, *K* (velocity and equilibrium constants) They are not followed by a full point except at the end of a sentence

Micro-organisms

The abbreviations marked * for generic names of micro-organisms may be used, but the full name must be given the first time the organism is mentioned in each paper

absolute	abs.	calculated	calc
acceleration due to gravity	<i>g</i>	Calorie (large, kilogram-calorie)	kg cal
acetyl, CH ₃ CO (in formulae)	Ac	calorie (small, gram calorie)	cal
†adenosinediphosphoric acid	ADP	candle power	c p
†adenosinetriphosphoric acid	ATP	centi- (prefix, 10 ⁻²)	c
†alanine	Ala	centimetre	cm
alkyl group (in formulae)	Alk	centimetre gram second	c g s
alpha-	α-	cerebrospinal fluid	c s f
alternating current	a c	* <i>Chromobacterium</i>	<i>Chromobact</i>
ampere	amp.	* <i>Clostridium</i>	<i>Cl</i>
ampere (with qualifying prefix or suffix only)	a	coefficient	coeff
analytical standard of purity	A R	coenzyme I, II	Co I, Co II
Ångstrom unit	Å.	compare	cf
ante meridiem	a.m	concentrated	conc
approximately	approx (or use about, not c)	concentration	concn
arginine	Arg	concentration (symbol, e g in specific rotation)	c
aryl group (in formulae)	Ar	configuration (amino acids and carbohydrates) (<i>Biochem J</i> 1948, 42, 1)	D-, L-, etc
atmosphere	atm	constant	const
†asparagine	(Asp NH ₂)	constant, equilibrium	<i>K</i>
†aspartic acid	Asp	constant, Michaelis	<i>K_m</i>
atomic weight	at wt	constant, velocity	<i>h</i>
* <i>Bacillus</i>	<i>B</i>	corrected (m p , for emergent stem)	corr
* <i>Bacterium</i>	<i>Bact</i>	* <i>Corynebacterium</i>	<i>C</i>
benzoyl, C ₆ H ₅ CO (in formulae)	Bz	cubic	cu
boiling point	b p , <i>pl</i> b p 's	curie	c
†British anti lewisite (2 3 dimercaptopropanol)	BAL	cycles per second	cyc /sec
British Pharmacopoeia	B P (with date)	†cysteic acid	(Cy SO ₃ H)
British Thermal Unit	B TH U		

†cysteine	(CySH)	foot candle	ft c
†cystine	(Cy ₂ S ₂)	freezing point	f p
decibel	db	gallon (Imperial)	gal
decomposition (m p)	decomp	†glutamic acid	Glu
degrees absolute (Kelvin)	°K	†glutamine	(Glu.NH ₂)
‡degrees Centigrade	°C	†glutathione	GSSG
degrees Fahrenheit	°F	†glutathione, reduced	GSH
density	<i>d</i>	†glycine	Gly
dextrorotatory (as prefix) (see also 'configuration')	<i>dextro</i> , (+)-	gram	g
†'dichlorodiphenyltrichloroethane', 1 1 1 trichloro 2 2-di(<i>p</i> chlorophenyl)ethane	DDT	gram calorie	cal
†3 4-dihydroxyphenylalanine	DOPA	gram ion	g ion
direct current	d c	gram molecule	g mol
dissociation constant (negative log of)	pK	Gram negative	Gram -
		Gram positive	Gram +
* <i>Eberthella</i>	<i>Eberth</i>	†haemoglobin	Hb
electrocardiogram	e c g	half wave potential (polarography) (cf <i>Biochem J</i> 42, 421)	<i>E</i> _½
electrode potential	<i>E</i>	height	ht
electrode potential, standard	<i>E</i> ₀	henry	H.
electrode potential, standard at constant pH	<i>E'</i> ₀	high frequency	h f
electroencephalogram	e e g	†histidine	H _{is}
electromotive force	e m f	horse power	h p
electron volt	eV	hour	hr
equivalent (weight)	equiv	hydrogen ion concentration	[H ⁺]
* <i>Escherichia</i>	<i>Esch</i>	hydrogen ion concentration (negative log of)	pH, <i>pl</i> pH's
ethyl, C ₂ H ₅ (in formulae)	Et	hydrogen pressure (in atmospheres) in equilibrium with oxidation reduction system	rH
Experiment (with reference numeral)	Exp	†hydroxylysine	Hylys
extinction (log <i>I</i> ₀ / <i>I</i>)	<i>E</i>	†hydroxyproline	Hypro
$E_{1\text{cm}}^{1\%} = E/cl$, where <i>c</i> is concn (% w/v) and <i>l</i> is length in cm		inch	in
farad	F	international unit	i u
feet per second	ft /sec	ionic strength	<i>I</i>
Figure (with reference numeral)	Fig	†isoleucine	Ileu
foot	ft	junior	jun

*† See introduction, pp 3-4

‡ Centigrade scale assumed in absence of indication, C to be used only when more than one scale is used

kilo (prefix, $10^3 \times$)	k	milli equivalent	m-equiv
kilovolt ampere	kVa	millilitre	ml
kilowatt hour	kWh	millimicron (10^{-9} metre)	m μ
<i>*Lactobacillus</i>	<i>Lb.</i>	§millimolar (concentration)	mM
laevorotatory (as prefix) (see also 'configuration')	<i>laevo</i> , (—)-	minimum, minute	min
†leucine	Leu	§molar (concentration)	M
litre	l	molecule	mol
logarithm, in text	log	molecular	mol
logarithm (base 10), in formulae	log	molecular extinction coeffi- cient = E/cl , where c is M concn and l is length in cm	ϵ
logarithm (base e), in formulae	ln	molecular optical rotation	$[M]_D^{20}$ etc
low frequency	l f	molecular proportion (in reactions)	mol
†lysine	Lys	molecular weight	mol wt
maximum	max	non-protein nitrogen (with definition)	N P N
median effective dose	ED ₅₀	§normal (concentration)	N
median lethal dose	LD ₅₀	normal (in organic com- pounds)	<i>n</i> -
mega- (prefix, $10^6 \times$)	M	normal temperature and pressure	N T P
melting point	m p, <i>pl</i> m p 's	number (in enumerations)	no
<i>meta</i> (in organic compounds)	<i>m</i> -	ohm	Ω
metabolic quotient of X (in μ l X/mg dry wt of bio- logical material/hr)	$Q_x \dagger$	<i>ortho</i> (in organic compounds)	<i>o</i> -
metabolic quotient of X (in any other units)	$q_x \dagger$	page, pages	p, pp
†methionine	Met	<i>para</i> (in organic compounds)	<i>p</i> -
methyl, CH ₃ (in formulae)	Me	parts per million	p p m
metre	m	<i>*Pasteurella</i>	<i>Past</i>
metre candle	m c	<i>*Penicillium</i>	<i>P</i>
micro- (prefix, $10^{-6} \times$)	μ	per (e g grams per litre)	/ (g / l)
microgram	μ g (<i>not</i> γ)	per cent	%
micromicro- (prefix, $10^{-12} \times$)	$\mu\mu$	population parameter corre- sponding to the s d of a sample (Not to be used ex- cept where reference is made to the population para- meters)	σ
micromicron (10^{-12} metre)	$\mu\mu$		
micromilli- (prefix, $10^{-9} \times$)	μ m		
micron (10^{-6} metre)	μ		
milli- (prefix, $10^{-3} \times$)	m		

*† See introduction, pp 3-4

† See notes on p 12

§ Separated by a hyphen (and no full stop) from a chemical formula or name following it,
e g m NaCl, n-NaOH, M solution

|| Percentage should be written in full, except in the Methods parts of papers and in Tables,
where the symbol '%' may be used for the noun

phenyl, C ₆ H ₅ (in formulae)	Ph	standard deviation	S D
†phenylalanine	Phe	standard error	S E
post meridiem	p m	standard error of the mean	S E M
potential difference	p d	* <i>Staphylococcus</i>	<i>Staph</i>
precipitate	ppt	* <i>Streptococcus</i>	<i>Strep</i>
probability of an event being due to chance alone (i.e. low values imply significant main effect)	P	substituents (variable, in organic compounds)	R, R'
†proline	Pro	sum	Σ
* <i>Pseudomonas</i>	Ps	temperature	temp
reciprocal centimetre	cm ⁻¹	tertiary (in organic compounds)	tert -
red blood corpuscles	R.B.C.	†threonine	Thr
refractive index	n (with wave length as subscript)	time	t
relative band speed (partition chromatography) Movement of band/movement of liquid surface above column (Martin & Synge, 1941, <i>Biochem J</i> 35, 1358)	R	†trichloroacetic acid	TCA
relative band speed (paper partition chromatography) Movement of band/movement of advancing front of liquid (Consden, Gordon & Martin, 1944, <i>Biochem J</i> 38, 224)	R _F	†1,1,1 trichloro 2,2,2 (p chlorophenyl)ethane	DDT
respiratory quotient	R.Q.	†tryptophan	Try
revolutions	rev	†tyrosine	Tyr
rontgen unit	r	uncorrected (m.p., for emergent stem)	uncorr
second (time)	sec	†valine	Val
secondary (in organic compounds)	sec -	value (e.g. iodine value)	val (e.g. I ₂ val)
sedimentation constant	S	vapour density	v d
†serine	Ser	vapour pressure	v p
species (sing. and pl.)	sp, spp	variety (e.g. botanical)	var
specific gravity	sp gr	velocity	v
specific heat	sp ht	viscosity	η
specific optical rotation	[α] _D ²⁰ , [α] _D , [α] ₄₄₀ ¹ etc	volt	V
square (e.g. square centimetre)	sq (e.g. sq cm)	volume	vol
		watt	W
		wavelength	λ
		wavelength of D line of sodium (other wavelengths in Å)	λ (as subscript)
		wave number (unit)	cm ⁻¹
		weight	wt
		yard	yd.

*† See introduction, pp 3-4

SPELLINGS, ETC., ADOPTED

<i>Adopted form</i>	<i>Remarks</i>
acetamido	<i>not</i> acetyl-amino
adenosinediphosphoric acid	see abbreviations
adenosinetriphosphoric acid	see abbreviations
adrenaline	
aetio (prefix)	roman, <i>no</i> hyphen
albumin	
(aliquot <i>not</i> used)	substitute 'sample' or 'portion'
alkali, pl alkalis	
<i>allo</i>	<i>italic, no</i> hyphen
amino acid	
ampoule	
analyse	other words from root <i>lyse</i> end in 'lyse'
aneurin	<i>alternative permitted</i> vitamin B ₁ , <i>not</i> thiamin
aneurinpyrophosphoric acid	<i>alternative permitted</i> cocarboxylase
anti-	hyphenate before compound words (e g anti blacktongue), but join with others (e g antiserum, antistreptococcal)
artifact	
ascorbic acid	<i>alternative permitted</i> vitamin C
asparagine	
baker's yeast	
benzamido	<i>not</i> benzoylamino
benzene (C ₆ H ₆)	<i>not</i> benzol, benzine <i>not</i> used
bis	roman
bivalent*	
body weight	
boiling point	<i>no</i> hyphen
brei	Anglicized, <i>pl</i> breis
brewer's yeast	
Buchner	
calciferol	<i>alternative permitted</i> vitamin D ₂
carbobenzyloxy- (CH ₂ Ph O CO O—)	<i>not</i> carbobenzoxo
cellophan	
choline	
cholinesterase	

* Latin prefixes will be used with words of Latin origin such as 'molecular' and '-valent', e g unimolecular', 'bivalent', 'ter', 'quadri-', 'quinque', 'sexa', etc, *not* 'monomolecular', 'divalent', 'tri', 'tetra', 'penta-', 'hexa-', etc

<i>cis</i>	italic, hyphen
co-carboxylase	
coenzyme I, cozymase	abbreviation Co I, <i>not</i> diphosphopyridine nucleotide, <i>not</i> DPN
coenzyme II	abbreviation Co II, <i>not</i> triphosphopyridine nucleotide, <i>not</i> TPN
connexion	
<i>cyclo</i>	italic, no hyphen
cytochrome c	
deoxy (prefix)	<i>not</i> desoxy
duodotyrosine	
dioxan	
disulphide group	<i>alternative permitted</i> S S group
dithionite	preferably $\text{Na}_2\text{S}_2\text{O}_4$, previously called hydro-sulphite, hyposulphite
egg white	
electron micrograph	<i>not</i> electron microgram
end point	
enzymic	<i>not</i> enzymatic
ethanol, ethanolic	<i>not</i> ethyl alcohol, <i>not</i> alcoholic
filter paper	
flavin adenine dinucleotide	
fluorimetry	measurement of fluorescence, <i>not</i> fluorometry
focusing	
fractions, e.g. one half, two thirds	use hyphen
freezing point	
gelatin	
glycerophosphoric acid	<i>alternative permitted</i> phosphoglycerol
guinea pig	
haem, protohaem	prosthetic group of haemoglobin
haematin, protohaematin	oxidized haem
haemochromogen	haem + base
haemoglobinometry	
hexosephosphoric acid	one word, <i>no</i> hyphen
hydrolysate	
hydrolyse	
(hydrosulphite, hyposulphite <i>not</i> used)	see dithionite
hydroxyl group	<i>alternative permitted</i> OH group, <i>not</i> hydroxy group

ice water

infrared

iodometric

ions

antimony, pentavalent, Sb^{5+} antimony, trivalent, Sb^{+++} calcium, Ca^{++} sulphate, SO_4^{--}

-ise, -ize

isobutanol

isoelectric

isoleucine

kephalin

light petroleum

lipid, phospholipin

litter mate

lyse

melting point

Messrs XYZ Ltd

methanol, methanolic

micro analysis

microcrystalline

micro Kjeldahl

micro organism

mustard gas

ninhydrin

non-

nor (in organic compounds)

ox

phenylglucuronide

phenylsulphuric acid

phlorrhizin

phospholipin

photomicrograph

one word, *no* hyphen

use termination 'ize' generally, but not 'lyze' for words ending in '-lyse' See Collins, *Authors' and Printers' Dictionary*

isoButanol at the beginning of a sentence

not petroleum ether

lipid material of biological origin which is soluble in fat solvents Lipoid not to be used Lipin as a suffix only, e.g. phospholipin, etc

spell with 's' not 'z', derived words analogously

no comma before 'Ltd' 'Messrs' used only in acknowledgements

not methyl alcohol

} general rule no hyphen except before vowel or before proper name

alternative permitted di 2 chloroethyl sulphide, special symbol *H*

hyphen

roman, *not* italic, *no* hyphen

domestic bovine species without sexual distinction, *not* beef

not phenol sulphate

potassium hydroxide	<i>not</i> caustic potash
pyridoxine, pyridoxal	<i>alternative permitted</i> vitamin B ₆
pyrrole	
quadrivalent	
quinol	<i>not</i> hydroquinone
quintivalent	
riboflavin	<i>alternative permitted</i> ribitylflavin, <i>not</i> lactoflavin, <i>not</i> vitamin B ₂
semi-	hyphen, except in chemical names, e.g. semicarbazide
separating funnel	
sexavalent	
sodium hydroxide	<i>not</i> caustic soda
stilboestrol (4,4'-dihydroxy $\alpha\beta$ diethylstilbene)	give systematic name first time, <i>not</i> called diethylstilboestrol
Student's <i>t</i> test	no literature reference
sulphydryl group	<i>alternative permitted</i> SH or thiol group
tap water	
tervalent	
thyroxine	
tocopherol	<i>alternative permitted</i> vitamin E
Tollens reagent	
toluene	<i>not</i> toluol
<i>trans</i>	italic, hyphen
trichloroacetic acid	see abbreviations
triosephosphate	
tryptophan	other amino acids have terminal 'e'
twofold	for numbers above ten thus, 11 fold
ultraviolet	one word, <i>no</i> hyphen
un ionized	
univalent	
water	<i>not</i> H ₂ O
water bath	
wavelength	
X ray	
χ^2 test	

ANALYSES, MELTING POINTS AND ROTATIONS

The standard forms for reporting these are as follows

The *new compound* (name in italics) had m p 175° (decomp), $[\alpha]_D^{25} + 17^{\circ} \pm 2^{\circ}$ in water (c, 1.6) (Found C, 40.8, H, 6.9, N, 11.5, OMe, 26.0 $\text{C}_8\text{H}_{10}\text{O}_6\text{N}_2$ requires C, 40.7, H, 6.8, N, 11.9, OMe, 26.3%)

The known compound had m p $178-179^{\circ}$. The mixed m p with an authentic sample kindly supplied by Dr Z was identical (Found C, 48.6, H, 6.1, OMe, 50.1. Calc for $\text{C}_{10}\text{H}_{18}\text{O}_7$, C, 48.4, H, 6.4, OMe 50.0%) *Alternatively* The known compound had m p $178-179^{\circ}$. The mixed m p with an authentic sample (m p $179-181^{\circ}$) prepared according to X & Y (1932) was $178-180^{\circ}$ (Found Calc for)

Notes

1 *Melting points* It is desirable to state whether m p 's are corrected or uncorrected for the emergent stem of the thermometer. This can often be done conveniently at the beginning of the experimental section.

2 *Specific rotations* It is desirable to give an estimate of the error involved.

3 *Percentages in elementary analyses* These should generally be given to one place of decimals only, since the second place is rarely significant.

METABOLIC QUOTIENTS

1 (a) The symbol Q_X may be used to represent metabolic quotients only in the units $\mu\text{l X/mg dry wt of biological material/hr}$. (b) If the metabolite X is a solid or liquid it is for the purpose of this convention considered as a gas at N.T.P., $1\mu\text{mol}$ of X being equivalent to $22.4\mu\text{l}$.

2 Metabolic quotients in any other units, e.g. $\mu\text{mol X/mg dry wt/hr}$, $\mu\text{l X/mg N/sec}$, may be represented by the symbol q_X .

3 The units for Q or q must be clearly defined at the first mention in each paper.

4 Production and removal of metabolites are indicated by quotients with + and - signs respectively. The signs may be omitted if no confusion can thus arise.

5 Aerobic or anaerobic conditions may be indicated by the superscripts O_2 and N_2 respectively, thus $Q_{\text{CO}_2}^{\text{O}_2}$, $Q_{\text{CO}_2}^{\text{N}_2}$, $q_{\text{CO}_2}^{\text{O}_2}$, $q_{\text{CO}_2}^{\text{N}_2}$. When measurements are made in another gas, this may be indicated by the appropriate superscript, e.g. $Q_{\text{CO}_2}^{\text{H}_2}$.

6 The substrate may, if necessary, be indicated in parentheses following the Q symbol, thus $Q_{\text{CO}_2}^{\text{N}_2}$ (pyruvate).

7 It is sometimes preferable not to use the special symbols Q_X or q_X , but to state units (e.g. $\mu\text{mol/g wet wt/hr}$) each time a metabolic quotient is mentioned.

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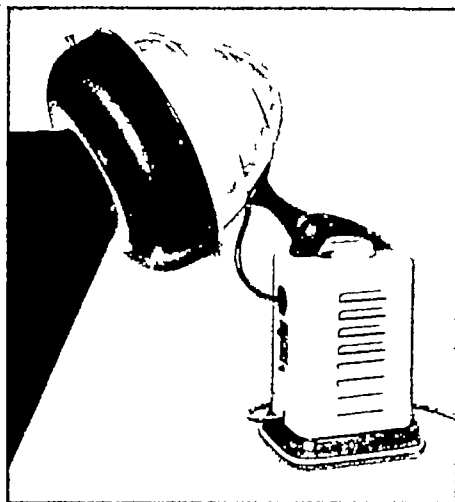


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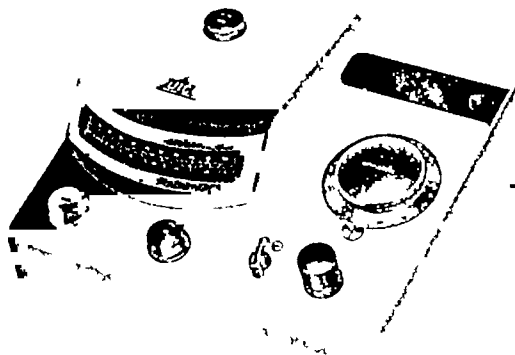
T 31

For Chemical Analysis

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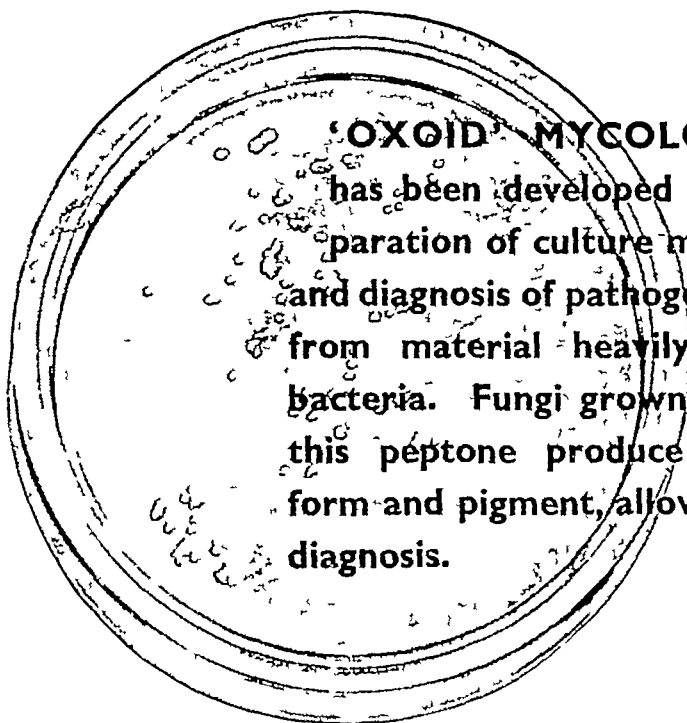


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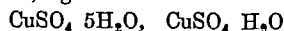
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The Distribution of Glycogen in the Liver of Rabbits

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(Received 28 March 1949)

Attention has recently been called by Gomori & Goldner (1947) to a strikingly uneven distribution of glycogen in the liver of rabbits. According to these authors, variations exceeding several 100% may be observed in simultaneous biopsies taken from the same or from different liver lobes.

In the present investigation, a survey of the distribution of liver glycogen has been carried out on rabbits submitted to a variety of experimental conditions. It has not been possible to confirm Gomori & Goldner's (1947) observations. Only relatively small intralobar and interlobar differences were observed, not exceeding those described in other animal species.

Only the distribution of glycogen will be considered here. The effects of the various experimental conditions on the glycogen content will be described elsewhere.

METHODS

Ten rabbits were chosen for the survey by sampling a very heterogeneous population, including fed and fasting (24 hr) animals, and fasted animals injected with anterior pituitary extract, or with glucose + insulin or with both (two animals in each group). Their liver glycogen content varied between 0.32 and 8.55%.

The animals were killed by intravenous injection of 500 mg sodium Evipan. Samples weighing approximately 100 mg were taken immediately after death and immersed in tared centrifuge tubes containing 2 ml of 30% (w/v) KOH. They were analysed according to Good, Kramer & Somogyi (1933), the final glucose determination being made by the method of Nelson (1944).

The sampling was made on the three main lobes of the liver: 1 = right lobe (+ gall bladder), 2 = middle lobe, 3 = left lobe. Three samples were taken from distant sites in each lobe. Three contiguous samples were further taken from one of the lobes for estimation of the error of determination.

Intralobar and interlobar variations were determined statistically by making a variance analysis of the results (Mather, 1946). In order to homogenize the variances from animals which were not immediately comparable, the analysis was made on the logarithms of the results, using natural logarithms. An estimation of the relative variations of the glycogen content throughout the liver was thereby obtained. It may be pointed out that the absolute differences between comparable results were found to increase with increasing glycogen contents, whereas the relative differences remained approximately constant.

RESULTS

The results of the variance analysis are summarized in Table 1.

Table 1. Variance analysis of distribution of liver glycogen

Origin of variance	Degrees of freedom	Sum of squares	Mean square	P	Relative s.d.* (%)
Rabbits + treatment	9	30.9722	—	—	—
Lobes	20	0.2558	0.0128	<0.001	8.6
Position in lobe	60	0.2332	0.0039	<0.001	6.4
Analytical procedure†	60	0.0092	0.0015	—	3.45

* Gives the equivalent of the logarithmic standard deviation from the mean, in percentage of the mean.

† Since only one of the nine determinations made on each animal was done in triplicate, the estimation of this factor could not be included in the general variance analysis, but was effected separately.

For the purpose of illustration, the results of the two experiments in which the largest discrepancies were observed have been recorded fully in Table 2.

Table 2. Distribution of liver glycogen

Rabbit no	Treat ment	Lobe	Glycogen content (g /100 g wet wt)				Mean of lobe	Mean of liver
			Site A	Site B	Site C			
8	Fasting (24 hr)	1	1 34	1 00	1 21	1 18	1 24	
		2	1 40	1 32	1 29	1 22		
			1 35					
			1 20					
		3	1 24	1 13	1 39	1 25		
		9	Fed	1	5 70	5 10		4 76
2	5 10			5 40	6 35	5 71		
	5 51							
	5 60							
3	4 71			4 52	4 93	4 72		

(a) *Error of determination.* This includes possible local differences between contiguous pieces of liver tissue. As is shown in Table 1, the standard deviation

from the mean of three results obtained on contiguous samples amounts to 3.45 % of the mean value

(b) *Intralobar distribution* When three pieces of liver are taken from distant sites in the same lobe, the variations are significantly larger than those arising only from determination errors (relative $s.d. = 6.4\%$, $P < 0.001$). These variations are, however, much smaller than those described by Gomori & Goldner (1947). It may be calculated that the probability of finding a local glycogen content more than 16 % higher or lower than the average content of the lobe is smaller than 0.01. Out of the sixty determinations of this kind which have been made, none shows a deviation of that magnitude and only three deviate more than 10 % from the mean lobar value, 10, 14.4 and 15.5 % respectively.

(c) *Interlobar distribution* Interlobar variations are significantly higher than the intralobar ones (relative $s.d. = 8.6\%$, $P < 0.001$), but here again the differences are not very important quantitatively. It may be computed from the data presented that a single glycogen determination, performed on 100 mg of hepatic tissue, would, under the conditions of our experiments, be representative of the average glycogen content of the whole liver with a standard error of 8.6 %. The probability that the result obtained would exceed the average content by more than 25 % is smaller than 0.01.

Closer analysis of the individual results shows that the heterogeneity of the lobar distribution is almost entirely due to the middle lobe (lobe 2), whose glycogen content is systematically higher than those of the two others, which are usually in close agreement (average increase for lobe 2 = 5.9 %, limits for $P = 0.01$ = 2.4 and 9.5 %).

(d) *Individual differences and differences due to treatment* It should be mentioned here that the results were first analysed separately for each individual rabbit and then by groups of two similarly treated animals. Comparison of the results of the

individual and group analyses showed that the relative distribution of glycogen in the liver was not significantly affected either by individual differences nor by differences due to the treatment. A bulk analysis of the homogenized variances of all the animals was thereby justified. Only the results of this analysis have been given in Table 1.

DISCUSSION

In view of the data presented here, there seems to be no reason to exclude the rabbit from studies involving determinations of liver glycogen. An analysis performed on three pieces taken at random in the three lobes of the liver should furnish a very satisfactory estimation of the average glycogen content of that organ.

It is difficult to reconcile these results with those of Gomori & Goldner (1947). However, the conclusions of these authors are partly based on histological evidence, and one may wonder to what extent microscopic heterogeneity is liable to affect the average glycogen content of a macroscopic sample of tissue.

SUMMARY

1 The distribution of glycogen in the liver of rabbits subjected to a variety of experimental conditions has been studied chemically and the results analysed statistically.

2 The distribution in the same lobe and in different lobes has been found to be significantly, though moderately, heterogeneous.

3 The relative $s.d.$ of a single determination with respect to the average glycogen content of the whole liver was found to be 8.6 % under the conditions of our experiments.

This work has been supported by a grant from the Lilly Research Laboratories.

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Prosthetic Groups of the Cytochromes Present in *Corynebacterium diphtheriae* with Especial Reference to Cytochrome *a*

By W A RAWLINSON* (Wellcome Travelling Research Fellow, 1947-8) AND J H HALE
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(Received 28 December 1948)

As a preliminary to investigations on porphyrin synthesis by *Corynebacterium diphtheriae* with various isotopically labelled compounds, we deemed it essential to study the prosthetic group of the cytochromes of this organism. The present paper reports the results so far obtained.

With *C. diphtheriae*, controlled improvement of toxin production has led to a corresponding increase in the production of free porphyrin, accurate adjustment of iron concentration is essential for the production of optimum amounts of toxin, even when other factors such as pH, nutrients, etc. are optimal. Pappenheimer (1947*a, b*) has shown that there is a parallel rise and fall of toxin and porphyrin excreted into the medium during cultivation under different conditions. The porphyrin was identified tentatively by Campbell Smith (1930) and Coulter & Stone (1931) and was characterized as coproporphyrin III by Gray & Holt (1947). A functional relationship between toxin and pigment was postulated by Pappenheimer (1947*a*) after he had obtained evidence to indicate that for every 4 atoms of iron added above the amount giving an optimum yield of toxin, there failed to appear in the culture fluid 4 mol of porphyrin and 1 mol of toxin. At such higher iron levels he found that cells contained greater amounts of a haemochromogen like substance. *C. diphtheriae* contains large amounts of cytochrome *b* and smaller amounts of *c* and *a* (Fujita & Kodama, 1934). In the present work the prosthetic group of cytochrome *b* has been identified as protohaem *no 9* and the prosthetic group of cytochrome *a*, although it has not been identified with any structure of established constitution, has been investigated in some detail.

EXPERIMENTAL

Measurement of absorption spectra. The wave lengths of absorption maxima were measured with a Beck Hartridge reversion spectrometer. In the case of intact cell suspensions, additional measurements were made by means of a Zeiss microscope eyepiece spectrometer.

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For the measurement of optical densities a Beckman photoelectric spectrophotometer was used. When dealing with solutions of pyridine haemochromogens, it was necessary to take the following precautions. The 1 cm glass cell was filled completely with solution and the lid fitted without the introduction of air bubbles. The solid $\text{Na}_2\text{S}_2\text{O}_4$ used as reducing agent was dissolved by rotating the cell. On account of the variability of different batches of $\text{Na}_2\text{S}_2\text{O}_4$, the minimum amount required to effect complete reduction was found by trial. In order to prevent turbidity appreciable excess of $\text{Na}_2\text{S}_2\text{O}_4$ was avoided. Measurements at wave lengths shorter than 400 $\text{m}\mu$ were not accurate as $\text{Na}_2\text{S}_2\text{O}_4$ shows a significant absorption in this region.

Determination of iron in haems. The o-phenanthroline method of Drabkin (1941) was found to be satisfactory, but it was necessary to use twice the amount of ascorbic acid prescribed by him. With that modification and measuring optical densities at 510 $\text{m}\mu$ (Beckman spectrophotometer) a straight line relationship was found to obtain for solutions containing 0-100 μg Fe/25 ml.

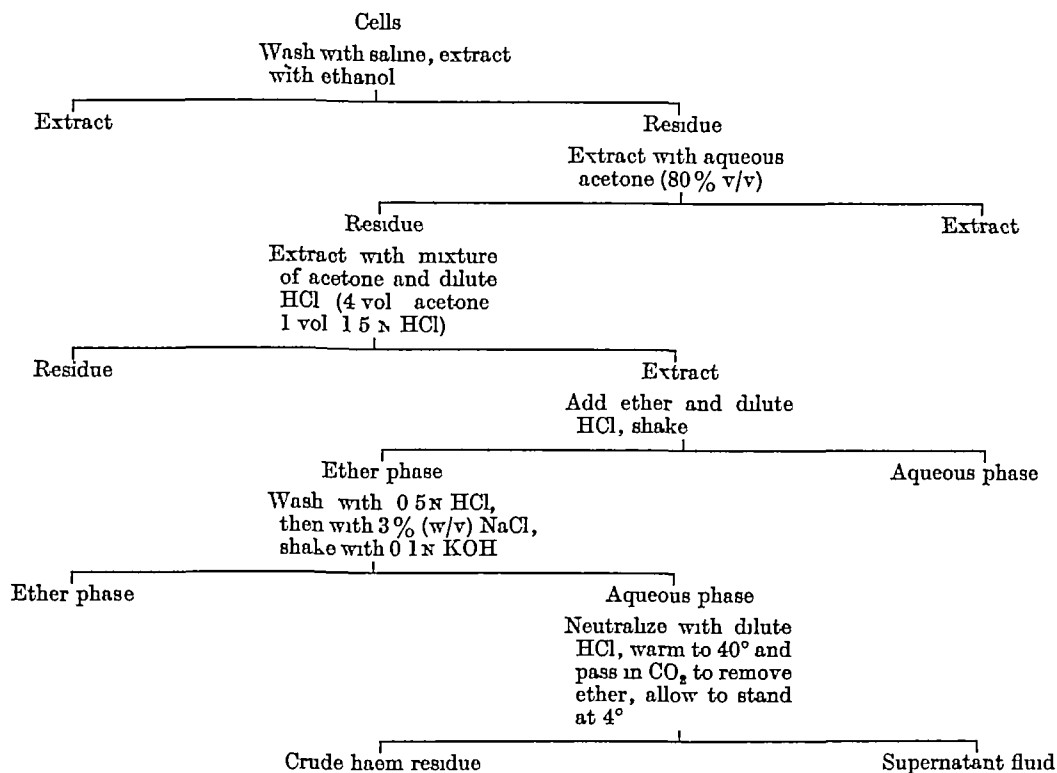
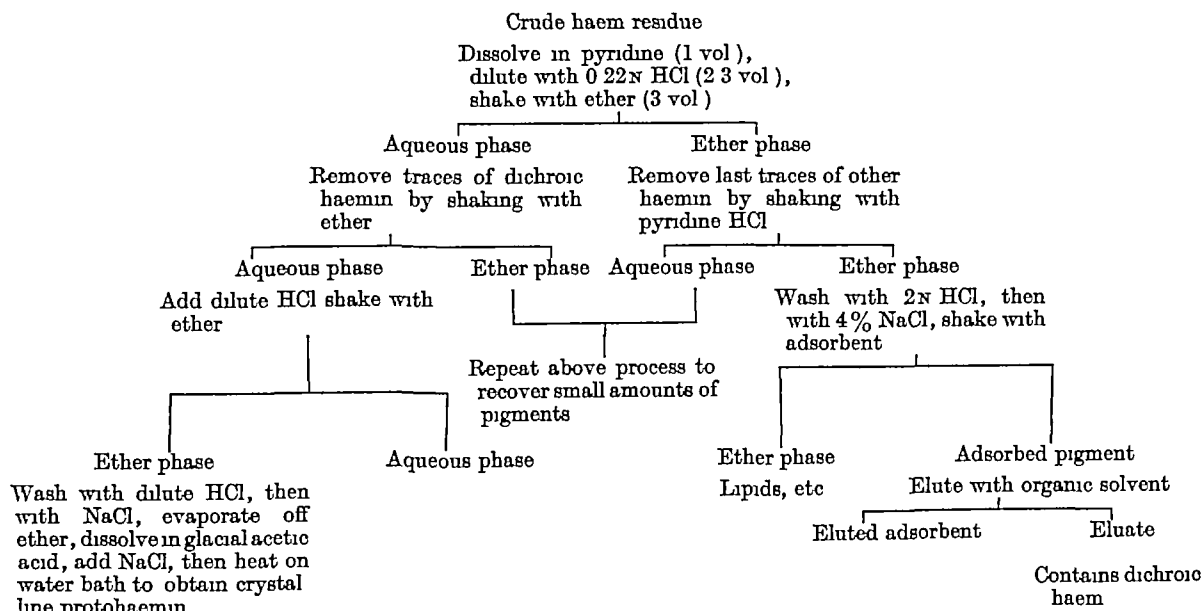
Cultivation of organisms. Two strains of *C. diphtheriae* were used: (a) Park Williams no. 8, substrain Toronto; (b) a variant of (a) supplied by Mr L. B. Holt of the Wright Fleming Institute of Microbiology, London, the organisms were grown on the casein hydrolysate medium described by Holt (1948), the Fe concentration being either that found necessary for optimum toxin production (0.14 μg /ml) or 10 times that amount. In one instance a large batch (180 l.) was grown on beef papain digest medium.

In all cases the cells were harvested by centrifuging and washed 3 times with saline before further treatment.

RESULTS

Spectroscopic investigation of intact C. diphtheriae cells grown at Fe level optimal for toxin production

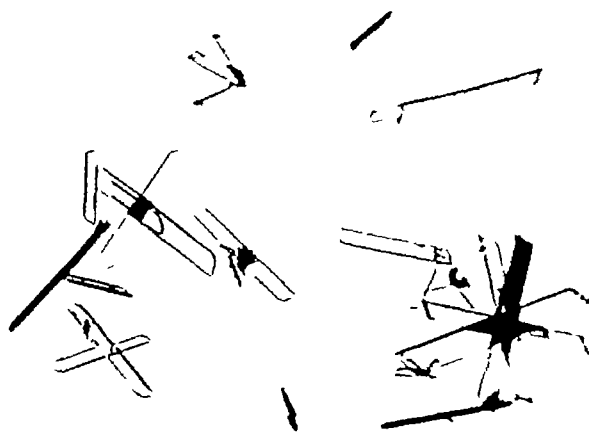
Aerated, thick suspensions of washed cells in phosphate buffer (pH 7.6) showed no definite absorption bands. Reduction with a small amount of $\text{Na}_2\text{S}_2\text{O}_4$ revealed bands at about 600, 565 and 532 $\text{m}\mu$, the first and last bands being of low intensity (figures given in italics indicate relatively weak absorption). The addition of pyridine, to give 25% (v/v) final concentration, brought about a shift of band positions to 590-580, 557 and 527 $\text{m}\mu$.

Fig 1 Separation of intracellular haems from *C. diphtheriae*Fig 2 Separation of two main haems from crude haem residues of *C. diphtheriae*

Cells grown at higher iron levels inhibitory to toxin production

In respect of spectral pattern these cells were similar to those at a lower Fe level but the band

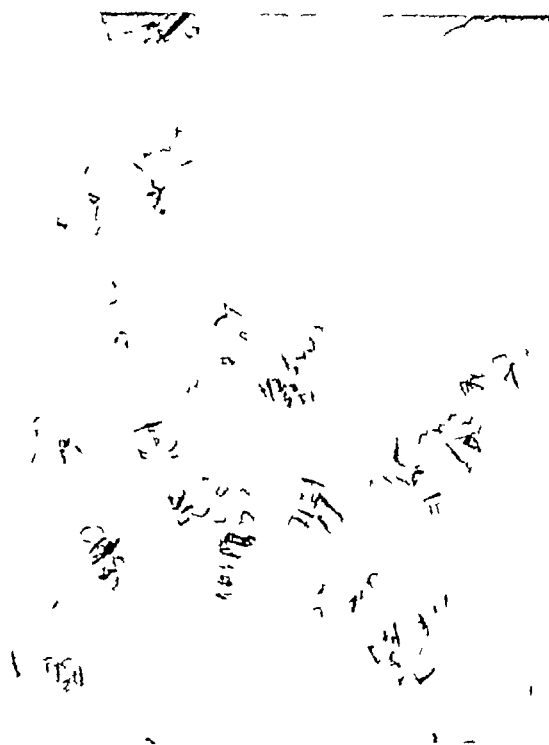
intensities were at least five times as great. This enabled us to measure accurately the band positions which were (in mμ) reduced cell suspension, 600, 568-554 (maximum at 563), reduced cell suspension, aerated (faint shading), 560-550, reduced



(a) Crystalline protohaemin from *C. diphtheriae* cells $\times 100$



(b) Pyridine haemochromogen crystals from protohaemin of *C. diphtheriae* cells $\times 100$



(c) Pyridine protohaemochromogen crystals $\times 100$

cell suspension and addition of pyridine, 599 582 (maximum at 590), 564 549 (maximum at 556), 522 (diffuse)

This result indicated the presence of two principal intracellular haem components and we concluded that there was an increase in cytochromes *b* and *a* complex in cells grown at higher iron concentrations (cf Pappenheimer, 1947*a, b*, Pappenheimer & Hendee, 1947)

Extraction and separation of intracellular haems from Corynebacterium diphtheriae

As attempts to extract the intact haemproteins were not successful, it was decided to extract the haem prosthetic groups by means of acid acetone. The flow sheet (Fig 1) illustrates the process used.

Tests on the extracts confirmed the presence of haems exhibiting haemochromogen bands at 587, 556 and 522 $m\mu$. The process outlined in Fig 2 gave clean separation if attention was paid to the number of washings given to each phase. The removal of final traces of unwanted pigments was checked, after each washing, by haemochromogen tests on small samples.

The dichroic haem was either adsorbed on charcoal and eluted with pyridine, or it was adsorbed on alumina, in which case elution with warm glacial acetic acid was found to be more effective.

Table 1 *The absorption spectra of protohaemin from cells of Corynebacterium diphtheriae compared with authentic protohaemin no 9*

(The two crystalline haemin samples were dissolved in acetone solution (0.3N with respect to HCl), transferred into ether, washed with 2N HCl and then with water before transfer into 0.1N KOH. The mixtures were diluted finally with 0.5 vol. of pyridine.)

Solvent	Protohaemin	
	<i>C. diphtheriae</i> ($m\mu$)	Authentic no 9 ($m\mu$)
Acetone HCl	642	642
	589*	590
	548	547
	505	505
Ether (HCl washed)	638	638
	586	586
	544	544
	503	503
KOH (0.1N)	615	615
	575	576
KOH (0.1N) + Na ₂ S ₂ O ₄	580	580
	543	544
KOH (0.1N) + Na ₂ S ₂ O ₄ + pyridine	556	556
	524	523
	487	486

* Figures in italics indicate relatively weak absorption.

Characteristics of the haem passing into the aqueous phase

The pigment from the aqueous phase, obtained in the form of Teichmann crystals (Pl 2*a*), gave with Takajama reagent crystals (Pl 2*b*) identical with those of pyridine protohaemochromogen (Pl 2*c*). Table 1 records a comparison between this haemin and authentic crystalline protohaemin no 9.

The quantitative spectral absorption curve (based on iron content, Fig 3) further illustrates the identity of the haemin with protohaemin. The iron content of the crystalline product was 96% of that of protohaemin prepared from ox blood and after recrystallization this value increased to 97%.

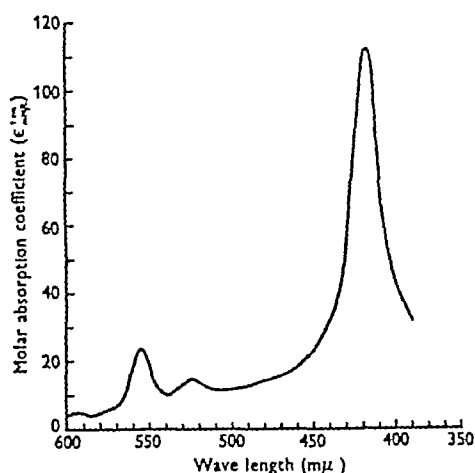


Fig 3 Absorption spectrum of pyridine protohaemochromogen from *C. diphtheriae*

Removal of iron. The SnCl₂-HCl method (Hamsik, 1931) was suitable for acetone solutions of the pigment, but for general purposes the ferrous acetate method of Warburg & Negelein (1932) was preferred. In Table 2 a comparison is made between

Table 2 *The absorption spectra of protoporphyrin derived from protohaemin of Corynebacterium diphtheriae cells compared with pure protoporphyrin no 9*

Solvent	Protoporphyrin	
	<i>C. diphtheriae</i> ($m\mu$)	Pure no 9 ($m\mu$)
Ether	632	632
	580	578
	537	537
	505	505
HCl (5 g HCl/100 ml.)	601	600
	580	579
	556	555
CHCl ₃ (from acidic solution)	605	604
	585*	584
	559	559

* Figures in italics indicate relatively weak absorption.

the porphyrin obtained and authentic protoporphyrin no 9

Conversion of the haemin to mesoporphyrin The hydriodic acid method of Fischer & Kögl (1924) was used. The mesoporphyrin was esterified with methanol saturated with hydrogen chloride, the ester purified in the usual way and crystallized four times from chloroform-methanol. A sample of protohaemin no 9 was treated similarly. The mesoporphyrin methyl ester crystals obtained were identical in form and gave similar melting points (207° uncorr.) Spectrometric comparisons are given for the free porphyrins and methyl esters in Table 3

Table 3 *The absorption spectra of mesoporphyrin derived from protohaemin of Corynebacterium diphtheriae cells compared with authentic mesoporphyrin no 9*

Solvent	Mesoporphyrin	
	<i>C. diphtheriae</i> (m μ .)	Authentic no 9 (m μ .)
Ether	623	623
	598	599
	578	580
	568	568
	527	527
	497	496
HCl (25 g HCl/100 ml)	594	593
	573*	574
	550	550
	—	—
CHCl ₃ † (from acidic solution)	595	595
	575	575
	551	552
	—	527
CHCl ₃ † (washed with NH ₃ soln., 12% diln of conc)	496	490
	620	620
	595	595
	576	576
	567	567
	532	532
	500	500

* Figures in italics indicate relatively weak absorption.

† After conversion into dimethyl ester

Characteristics of the haem from the ether phase

Solutions of this pigment were dichroic, concentrated solutions being red, while dilute solutions were yellow green. The pyridine haemochromogen test on this haem showed a strong band at 587, a weak band at 553 and a very faint band at 525 m μ (see Fig. 4). This indicated that the pigment contained more than one component. The 553 and 525 m μ bands were observed to increase in intensity relative to the 587 m μ band when solutions of this fraction were left standing. The absorption curve was therefore assumed to be the resultant of a main component with absorption maximum at 587 m μ , and a product derived from it with bands at 553, 525 and about 418 m μ . The addition of cysteine

hydrochloride to a solution of the pyridine haemochromogen mixture caused the slow disappearance of the 587 m μ band and a simultaneous appearance of extra bands close to the 553 and 525 m μ bands.

Removal of iron The method of Warburg & Negelein (1932) was used to remove the iron from this fraction and an inspection with the reversion spectrometer clearly revealed the presence of two sets of porphyrin bands. These were (pyridine solution) I (a) 645, (b) 627, II, 593 581, III (a) 559, (b) 541, IV, 505 m μ . Order of intensity III (a), II, IV, I (a), I (b), III (b). A partial separation of the two components was achieved by extraction from ether solutions by means of hydrochloric acid solutions of different concentration. Neither porphyrin was readily extractable by solvents less acidic than 3N HCl.

Comparison of the a component from Corynebacterium diphtheriae cells with that from heart muscle

At this stage it was decided to reconsider the value of further investigations on the pigments in this ether fraction. Although partial separation of the two porphyrins was possible by differential acid extraction, the extra manipulations involved and the difficulties experienced in eliminating reactive substances carried over from the original extracts led us to attack the problem in a different manner. The justification for this decision will be discussed below, but for the sake of continuity a brief comment is needed. The pigment responsible for the 587 and 430 m μ pyridine haemochromogen bands was assumed to be derived from the cytochrome *a* complex. It was therefore considered worth while to investigate another tissue (ox-heart muscle) known to be rich in this component.

Modifications of extraction and separation processes used in isolating the dichroic haem a from heart muscle Ox hearts were minced after freeing them from fatty and ligamentous tissue and then given either a preliminary dehydration with ethanol or extracted directly with acetone. Either anhydrous or 80% (v/v) aqueous acetone was equally satisfactory. A mechanical press was used to remove fluid, and extractions were continued until yellow pigments were no longer apparent (normally three extractions). The intracellular haems were extracted as before, care being taken to compensate for acid bound to tissue material during the first extraction. We have used successfully for first extractions mixtures with final concentrations 0.3N with respect to HCl and ranging from 80 to 98% aqueous acetone. With subsequent extractions less HCl was required to maintain acidic conditions in the extraction fluid. The greater part of the haem pigments was extracted rapidly, prolonged treatment being necessary to recover further significant amounts. Extractions were made at 4° to minimize the risks of decomposition.

From extracts containing little water and HCl, the pigments were precipitated by addition of saturated sodium acetate solution. The crude haem residue obtained by washing the precipitate with acetone and then dilute acetic

acid was now ready for solution in pyridine and isolation. This technique was most useful in removing lipid matter. If the extracts would not yield precipitated haem, by such direct treatment, the pigments were transferred into ether by the process outlined in Fig. 2, transfer into dilute alkali being found unnecessary.

The aqueous pyridine phase contained at least one other haem compound in addition to protohaem, as haemochromogen tests showed two additional bands at wave lengths slightly lower than the α and β bands of protohaemochromogen. The dichroic haem was adsorbed by passing the washed ether phase through a column of alumina after which the alumina was washed well with fresh ether. By stirring the dry residue with warm glacial acetic acid (50–60°) the haem was eluted to give a dark brown solution. In this condition the pigment remained stable even after long standing at room temperature.

Characteristics of haem a isolated from ox heart muscle. The results of spectroscopic investigations of this haem are summarized in Table 4.

Pyridine haemochromogen a. The quantitative absorption curve illustrated in Fig. 4 was obtained by dissolving a sample of the dichroic haem, containing 34.9 μg Fe in 0.25 ml pyridine (A.R.) and 12.5 ml 0.1N NaOH and adding $\text{Na}_2\text{S}_2\text{O}_4$. This solution was diluted to 25 ml with distilled water and measurements were made with a Beckman spectrophotometer. For wavelengths below 474 $\text{m}\mu$, 2.0 ml stock solution was diluted to 7.0 ml with control solution (25% pyridine, 0.05N NaOH) and in this way maximum density values above 0.7 were avoided. This solution shows only a single band at 587, but when left standing for several hours in the presence of cysteine, was found to undergo change in the same way as the haem obtained from *C. diph-*

theriae cells did. The 587 $\text{m}\mu$ band faded and was replaced by two bands at 553 and 525 $\text{m}\mu$.

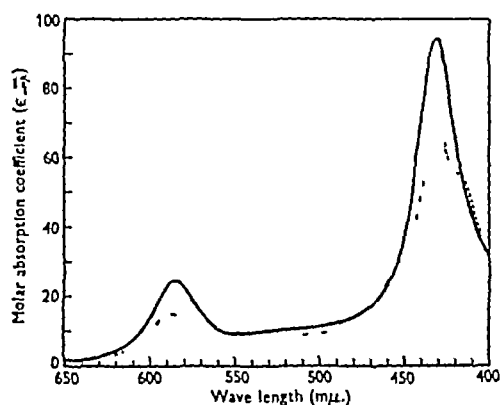


Fig. 4 Absorption spectra of pyridine haemochromogens. Heart haemochromogen a —, *C. diphtheriae* haemochromogen a - - Solvent, 3.11 M pyridine in 0.05N NaOH.

Removal of iron. The method of Warburg & Negelein (1932) was used for the preparation of the porphyrin. In Table 5 are presented the absorption maxima and orders of intensities of the pigment in various solvents. Neutral organic solutions were coloured reddish when the porphyrin concentration was high and yellowish green in dilute solution. The porphyrin was insoluble in light petroleum (b.p. 40–60°).

From the data presented it was concluded that haem a isolated from ox heart muscle was identical with that from *C. diphtheriae* cells, but, fortunately,

Table 4 Absorption spectra of haem a derivatives

Compound	Solution	Absorption bands			Order of intensity
		I ($\text{m}\mu$)	II ($\text{m}\mu$)	Soret ($\text{m}\mu$)	
Haem a	KOH (0.17N)	635	577	—	I, II
Haem a	Alkali + $\text{Na}_2\text{S}_2\text{O}_4$	578	531	—	I, II
CO haem a	Alkali + $\text{Na}_2\text{S}_2\text{O}_4$ + CO	591	542	—	I, II
Pyridine haemochromogen a	Alkali + pyridine + $\text{Na}_2\text{S}_2\text{O}_4$	587	—	430	—

Table 5 Absorption bands of ox heart dichroic porphyrin a^*

Solvent	I ($\text{m}\mu$)	II ($\text{m}\mu$)	III ($\text{m}\mu$)	IV ($\text{m}\mu$)	E.A.† ($\text{m}\mu$)	Order of intensity
CHCl_3	648	590	561	520	450	III, II, IV, I
Benzene	647	591	561	519	451	III, II, IV, I
Glacial acetic acid	641	591	560	520	454	III, II, IV, I
Ether	642	587	556	515	439	III, II, IV, I
Pyridine	648	589	559	527	444	III, II, IV, I
Pyridine ether	647	589	558	517	455	III, II, IV, I
HCl (10 g/100 ml)	618	563	—	—	—	II, I
KOH (0.1N)	582	505	519	—	447	II, III, I,

* From measurements with the Beck Hartridge spectrometer.

† End absorption.

the former contained none of the secondary product seen in the material isolated from the micro organisms

Experiments to confirm the suitability of isolation procedures

In order to test the possibility that the dichroic pigment might have been an artifact derived from protohaem, similar processes of extraction and separation were performed on aqueous acetone solutions (acid, neutral and alkaline) of pure protohaemin. After these solutions had been allowed to stand for 2 days at room temperature, with frequent shaking in air, no haem component was detected with characteristics similar to the above dichroic pigment. Whole ox blood, when subjected to the processes, did not yield any pigment with preference for the ether phase.

Further experiments with haem a

The more readily obtainable product from ox heart muscle was used in these investigations.

Effect of carbon monoxide on haem a On bubbling CO through an alkaline solution of haem a reduced with $\text{Na}_2\text{S}_2\text{O}_4$, there was an immediate shift of the absorption spectrum to give bands at 591 and 541.5 μ .

Effect of hydroxylamine on haem a A solution of Na_2CO_3 was added to a pyridine solution of the haem to give a 20% (v/v) final concentration of pyridine. The addition of a few crystals of hydroxylamine hydrochloride followed a few minutes later by the addition of $\text{Na}_2\text{S}_2\text{O}_4$ resulted in the appearance of a haemochromogen spectrum with maxima at 570 and 533 μ (cf. haemochromogen spectrum of original haem 587 μ).

Further experiments on the porphyrin obtained from haem a

Effect of cysteine on porphyrin a The addition of cysteine hydrochloride to a warm pyridine solution of the porphyrin, containing anhydrous Na_2CO_3 , resulted in a shift of band positions to I, 634, II, 578, III, 546, IV, 508 μ . (order of intensity III, IV, II, I)

Effect of hydroxylamine on porphyrin a The porphyrin was dissolved in pyridine and anhydrous Na_2CO_3 added. The addition of a few mg of hydroxylamine hydrochloride was followed by an immediate shift of band positions, even at room temperature, and after this change no further shift was observed by heating at 100° for 1 hr. The mixture was diluted with water and shaken with ether. The pigmented ether phase was washed well with 1% (w/v) HCl and finally with water to remove traces of acid. The spectrum in washed ether was I, 639, II, 582, III, 547, IV, 509 μ . End absorption (E.A.) 444 μ (order of intensity III, IV, II, I). The addition of an equal volume of pyridine gave the following bands I, 637, II, 582, III, 549, IV, 509 μ .

In Fig 5 this oxime reaction at 15° is demonstrated by measurements made in the Beckman spectrophotometer. The solutions used to obtain the curves were identical except for the addition to one of a small amount of hydroxylamine hydrochloride.

Copper complexes of porphyrin a The copper complex was formed by adding a small amount of cupric acetate to a solution of the free porphyrin in glacial acetic acid and heating in a boiling water bath for 5 min. The absence of red

fluorescence under ultraviolet radiation indicated completion of the reaction. In Table 6 are shown the positions of absorption band maxima in various solvents.

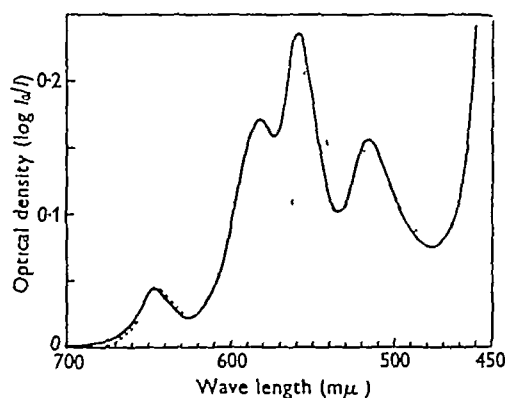


Fig 5 Absorption spectra of heart porphyrin a derivatives. Heart porphyrin a —, heart porphyrin a oxime - - Solvent pyridine

Table 6 Absorption bands of copper complex of heart dichroic porphyrin a

Solvent	I (mμ.)	II (mμ.)	III (mμ.)	Order of intensity
Glacial acetic acid	597	552	—	I, II
Pyridine	599	553	—	I, II
Ether	594	548	525	I, II, III
Pyridine ether	597	552	530	I, II, III

The addition of hydroxylamine hydrochloride to a pyridine solution of the copper complex containing anhydrous Na_2CO_3 brought about a shift of band positions within a few minutes at room temperature, to I, 587, II, 542, III, 504 μ . E.A. 442 μ . (order of intensity I, II, III)

Esterification of porphyrin a The porphyrin a was allowed to stand 2 days at 4° in methanol saturated with HCl, absorption bands I, 605, II, 563 μ . (order of intensity II, I). The pigment was taken into CHCl_3 after dilution with water. The CHCl_3 solution was extracted 4 times with 2N NH_4OH , 3 times with 10% (w/v) NaCl and then run through a dry filter paper. After evaporation to dryness and solution of the residue in pyridine ether solution, bands were observed at 648, 630, 589, 560, 510 μ . The presence of the band at 630 μ and the general appearance of the band pattern indicated that a small amount of the pigment had undergone change. After extraction of this solution with 10% (w/v) HCl to remove pyridine and traces of modified porphyrin the absorption bands were measured again after washing with water and dilution with an equal volume of pyridine (646, 589, 558, 516 μ .) cf. Table 4. The treatment of porphyrin a (dissolved in ether) with diazomethane did not appear to produce any modified pigment and so was considered preferable to the method described above for esterification.

DISCUSSION

The spectrometric tests made on intact cell suspensions of *C. diphtheriae* demonstrate the effect of iron concentration upon cytochrome content. The faint

absorption bands seen in washed cells grown at the lower iron levels agreed in wave length with the three main bands of cells grown at higher levels. It is concluded that, although the main types of haem constituents bound within the cells are independent of iron concentration, the quantity produced is decreased by low concentrations of iron and such inhibition is associated with an increased excretion of coproporphyrin III. A five to ten fold increase of intracellular haem components seems to take place when cells are grown at iron levels sufficient to reduce toxin and coproporphyrin production in the medium from optimal to insignificant amounts.

The absorption patterns and mean positions of wave lengths of maxima seen in reduced cell suspensions demonstrated the presence of cytochrome components. The intense band at $562\text{ m}\mu$ and the band of lower intensity at $531\text{ m}\mu$ resemble the α and β bands of cytochrome *b* except that they are shifted to values slightly lower than the *b* bands of some other tissues. Other micro organisms show similarly low values. Small differences in band positions measured by direct vision spectroscopy, may not always be significant, and confirmatory specific functional tests are needed before they are attributed to new components.

The absence of cytochrome *c* was apparent by examination of the $562\text{ m}\mu$ band. The general appearance of this band with its edges at 568 and $554\text{ m}\mu$ makes it difficult to assume any contribution from the $550\text{ m}\mu$ α band of cytochrome *c*. This was further confirmed by comparison of *C. diphtheriae* cells with other micro organisms known to possess cytochrome *c*. The absorption band at $600\text{ m}\mu$ indicates the presence of the cytochrome *a* complex. Experiments similar to those used in general cytochrome studies allowed us to confirm the presence of cytochrome oxidase (Pappenheimer, 1947b).

The shift of absorption bands to shorter wave lengths on the addition of pyridine to reduced cell suspensions results from a displacement, by this base, of the normal protom moieties of the cytochrome. The $587\text{ m}\mu$ band represents the pyridine haemochromogen complex of the *a* component and the bands at 557 and $526\text{ m}\mu$ that of the prosthetic group of cytochrome *b*. The positions of the latter bands coincide with the α and β bands of pyridine protohaemochromogen. Pyridine coprohaemochromogen exhibits bands at 548 and $518\text{ m}\mu$, and as these cannot be demonstrated in 'high iron' cells the iron complex of coproporphyrin III must be absent. It was concluded from the above tests that at least two cytochrome components were present (viz *a* and *b*) with dissimilar prosthetic groups. The identification of the major component as protohaem no. 9 is confirmed by all tests on the crystalline product isolated from the aqueous phase. Quantitative measurements on acid acetone extracts, after con-

version of the pigment into pyridine haemochromogen or protoporphyrin, gave values equivalent to $1.0\text{--}1.1\text{ mg}$ protohaem/l of culture. As the $587\text{ m}\mu$ pyridine haemochromogen band of component *a* (see p 254) is known to possess a similar extinction value to protohaemochromogen, its low intensity compared with that of the $557\text{ m}\mu$ absorption band of the latter component in intact cells must mean that it represents a small fraction of the total intracellular haems.

The dichroic pigment in the ether phase (from *C. diphtheriae* cells) was composed of two haems. The following facts point to derivation from one original component: both haems showed preference for the ether phase, the porphyrins derived from them both possessed high acid numbers, and on standing in solution the component with haemochromogen bands at 553 and $525\text{ m}\mu$ increased at the expense of the predominant $587\text{ m}\mu$ component. The reactions with hydroxylamine and cysteine also supported this conclusion.

As the interpretations of data were complicated by the presence of a product derived from the original haem it was decided that further studies to ascertain the constitution of the pigment might be facilitated by preparation of material from a more convenient source than *C. diphtheriae* cells. The haem *a* from ox heart muscle possessed spectroscopic characteristics similar to those of the haem from *C. diphtheriae* cells and (presumably due to smaller amounts of reactive impurities) was far more stable during its separation, and was therefore the material of choice. Lipid material accompanied the haem so closely during extraction that the possibility that some of it is an integral constituent cannot be excluded (cf. chlorophylls *a* and *b*).

In experiments with heart muscle our primary aim was to study the dichroic haem *a*. One pigment with haemochromogen α and β bands at wave lengths slightly shorter than protohaemochromogen was found in small amounts in the aqueous acid acetone phase after extraction with ether. Further work is in progress to establish the nature and significance of this pigment. Although cytochrome *c* is not dissociated by treatment with acetone and hydrochloric acid it is not impossible that some pigment is dissolved. The great affinity of the *c* component for aqueous phases could then explain the presence of a haem derivative in the acid acetone layer.

Although it had been concluded that the pigment responsible for the 587 and $430\text{ m}\mu$ absorption bands, in pyridine extracts of *C. diphtheriae* cells and ox heart muscle, was the prosthetic group of the cytochrome *a* component it was necessary to prove that this haem had not arisen as a degradation product of protohaem. Attempts to produce degradation products of protohaem under the conditions applicable during extraction procedures failed (cf. Negelein,

1932*a, b*, Negelein, 1933, Roche & Benevent, 1936) The claim was made in the paper last mentioned that another derivative, obtained from heart muscle by a different process and possessing pyridine haemochromogen bands at 587, approx 554, 530 and 425 $m\mu$, was, in fact, the true haem *a* from cytochrome *a*. An inspection of the absorption curve of this pigment, however, leads us to suggest that the product of Roche & Benevent (1936) was a mixture composed of one component with a band at 587 $m\mu$ and another component with bands at approx 554 and 530 $m\mu$, similar to the haemochromogen of the pigment found to accompany haem *a* in preparations from *C diphtheriae* cells. For reasons stated earlier we consider this material (maxima 553, 525 $m\mu$) to have been derived from the haem *a* by combination with substances accompanying the latter during extraction (compare the action of cysteine on haem *a*). The stability of our haem *a* to acid offers another distinct contrast with the product described by these workers. The 'kryptoporphyrin' obtained by the transformation of protoporphyrin (Negelein, 1932*b*) possessed a lower acid number than the dichroic pigment isolated by us from *C diphtheriae* cells and heart muscle.

The α band position of the carbon monoxide haem *a* complex (591 $m\mu$) is reminiscent of the position of one band of the light sensitive carbon monoxide compound of Warburg's respiratory enzyme (cytochrome oxidase of Keilin). At the present time it is considered that cytochrome *a* and cytochrome oxidase are related at least in the nature of their prosthetic groups.

The quantitative absorption curve of pyridine haemochromogen *a* (Fig. 4) shows that it differs considerably from the normal type of haemochromogen. In general, α and β bands are observed in the visible region of the spectrum, and even in the case of chlorocruorin haemochromogen we find, in addition to an α band at 583 $m\mu$, a second, weaker band at 542 $m\mu$. The extinction coefficients of the band maxima at 587 and 430 $m\mu$ of haemochromogen *a* approach those of the majority of haemochromogens, but the greater width of the former band makes it difficult to appreciate in direct vision spectroscopy that such is the fact.

The effect of cysteine on the pyridine haemochromogen *a* is similar to that reported for many haem derivatives (Fischer & Mittermaier, 1941). Fischer & Mittermaier concluded from experiments with derivatives of chlorophyll *b* that the shift was due to the presence of a reactive aldehyde group which with cysteine gave compounds of the mercaptal acetal type. The experiments we have reported with hydroxylamine and cysteine on both haem *a* and its porphyrin therefore indicate the presence of a carbonyl group in our material. There is the possibility that the carbonyl groups are ketonic, but

the rapidity of the addition reactions suggests that labile aldehyde groups are involved.

The reaction of haem *a* with hydroxylamine results in the formation of an oxime, the pyridine haemochromogen bands of which are situated at 570 and 533 $m\mu$. In the case of chlorocruorin haemochromogen (1 3 5 8 tetramethyl-2 formyl 4-vinyl porphyrin 6 7 dipropionic acid iron complex), oxime formation shifts its 583 $m\mu$ band to 557 $m\mu$, i.e. identical with the haemochromogen of protoporphyrin no. 9, and, on this fact, it is assumed that the $-\text{CH}=\text{NOH}$ group exerts an effect similar to that of a $-\text{CH}=\text{CH}_2$ group (compare the haemochromogens of mesoporphyrin and protoporphyrin). The shift of band position shown by haemochromogen *a* on conversion to oxime is 17 $m\mu$, a value which is similar to the shift (16.5 $m\mu$) shown by formylpyrroporphyrin no. 15, but much lower than in the case of chlorocruorin haemochromogen (26 $m\mu$). This comparison indicates that haemin *a* must possess structural groups (other than such active carbonyl groups) either different in type or in their arrangement around the nucleus from those found in protohaemin or chlorocruorohaemin.

The method used for preparing porphyrin *a* appeared to involve no significant modification of substituent groups, as the re-introduction of iron produced the same type of haemochromogen spectrum as that of the original substance. We could not find any evidence that the reaction with hydroxylamine proceeds in two stages, and so it must be assumed that in porphyrin *a* there is only one labile aldehyde group/mol. of pigment. The curves of Fig. 5 illustrate the differences between the oxime and original porphyrin *a*. All bands are shifted towards the blue, bands I and III remain unchanged in extinction values, but bands II and IV reverse their relative intensities. The oxime shows the band relationships (III, IV, II, I) characteristic of the 'rhodo type' of porphyrin.

SUMMARY

1. The cells of *Corynebacterium diphtheriae*, when grown at iron levels sufficient to inhibit the excretion of toxin and coproporphyrin, showed a five to ten fold increase of haem components over the level present when toxin and porphyrin excretion are maximal. The presence of cytochrome oxidase (cytochrome *a* complex) and cytochrome *b* were confirmed spectroscopically.

2. Methods for the separation of the two main haem prosthetic groups are described. Crystalline protohaemin, which is assumed to be derived at least in part from cytochrome *b*, and a dichroic haem, presumably from the cytochrome *a* complex, were obtained.

3. The dichroic haem was identified with the

haem of cytochrome *a* of ox-heart muscle and further studies on its characteristics were made on material from this more accessible source

4 The general properties of this haem and its reactions with pyridine, cysteine, carbon monoxide and hydroxylamine were investigated

5 The free porphyrin, like the haem from cytochrome *a*, reacted with cysteine and hydroxylamine

6 The results of the tests described indicate that this haem possesses at least one aldehyde group

One of us (W A R) is grateful to the Trustees of the Wellcome Foundation for a Travelling Fellowship held during the course of this work, and also for a grant for chemicals. Other expenses were defrayed from a research grant to the Department of Bacteriology from the Medical Research Council

We wish to thank Messrs G D Clark and L B Holt of the Wright Fleming Institute and Drs C G Pope and F V Lingwood of the Wellcome Physiological Laboratories for large scale cultures, and Miss K Hale for technical assistance

Our thanks are also due to Prof C Rimington and Prof Wilson Smith for their continued interest and encouragement at all times in this work

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The Oxidation of Manganese by Plant Extracts in the Presence of Hydrogen Peroxide

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(Received 28 January 1949)

Manganese is known to be an essential micronutrient of plants, although its function is unknown. It apparently plays a part in plant respiration, Lundegårdh (1939) found that the oxygen uptake of manganese deficient wheat roots was raised by 155-470% by the addition of 5×10^{-6} M manganese chloride. Such an effect might be brought about by the activation of certain enzyme systems, e.g. arginase (Waldschmidt Leitz & Purr, 1931), phosphoglucomutase (Cori, Colowick & Cori, 1938), leucylpeptidase (Berger & Johnson, 1939) or by a system in which Mn^{++} undergoes alternate oxidation and reduction. It has been shown that soil microorganisms can oxidize Mn^{++} (e.g. Beijerinck, 1913, Gerretsen, 1937, Leeper & Swaby, 1940, Mann & Quastel, 1946). No satisfactory evidence has been put forward to show that Mn^{++} is oxidized in higher plants and the present work was undertaken to investigate whether such oxidation does take place

MATERIALS AND METHODS

Preparation of plant extracts The roots were scrubbed free from soil and minced twice in a meat mincer. The mince was weighed and the juice squeezed out by hand through madapollam. Water to one quarter of the original weight of the mince was added to the residue, which was then ground in a mortar with sand and squeezed through madapollam. This treatment was repeated twice. The extracts were combined and filtered through Whatman no. 1 filter paper, and stored in a refrigerator. Most of the work was done with horse radish (*Cochlearia armoracia*), it was found that extracts of this root showed little loss of activity over a period of several weeks. In view of the possibility of contamination, however, fresh extracts were frequently made. Extracts of other roots were made and used on the same day. This method was originally adopted with horse radish as benzidine H_2O_2 tests showed satisfactory extraction of peroxidase.

Catalase preparation The caps of the Basidiomycete, *Marasmius oreades*, were ground with sand, squeezed through madapollam and centrifuged at 3500 rev./min.

(1500 g) for 0.5 hr. The catalase activity (Katalasefähigkeit) of the supernatant liquid, estimated by the method of Sumner & Somers (1943), was about 80. The catalase activity diminished rapidly and consequently the preparation was made and used on the same day.

Peroxidase preparation. The peroxidase preparation was made by the method of Keilin & Mann (1937) from horse radish. After precipitation with $(\text{NH}_4)_2\text{SO}_4$, followed by fractional precipitation with ethanol and further purification with tricalcium phosphate gel, a preparation of Purpurogallinzahl (PZ) 290 was obtained. The PZ, i.e. mg purpurogallin formed by 1 mg enzyme preparation in 5 min from pyrogallol and H_2O_2 under fixed conditions, was determined by the method of Keilin & Mann (1937).

Preparation of hydrated manganese dioxide. The suspension of hydrated MnO_2 was prepared by the method of Heintze & Mann (1949), which is based upon the dismutation of manganopyrophosphate in alkaline solution. The valency, as determined from the Mn content of the preparation and its oxidizing capacity towards oxalic acid, was 4.08.

Manometric measurements were carried out in the Warburg apparatus at 25° . The volume of the reaction mixture was 3 ml and KOH was present in the centre cup unless otherwise stated.

EXPERIMENTAL AND RESULTS

Colorimetric evidence of manganese oxidation

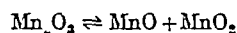
In preliminary experiments with certain types of root extracts colour reactions were obtained which indicated the formation of manganic complexes. The addition of MnSO_4 and H_2O_2 to the extracts in the presence of citrate at pH 7 caused the development of a greenish yellow colour, while in pyrophosphate at this pH a pink colour was obtained. On acidification of the pyrophosphate solution the colour changed to a brighter pink, both the pink and greenish colours were rapidly discharged by the addition of acid H_2O_2 or hydrazine sulphate. These reactions are typical of manganopyrophosphate and citrate complexes. The colour reactions were not given by extracts which had been heated for 5 min in a boiling water bath, nor were they given in absence of H_2O_2 or Mn^{++} .

Reaction mixtures consisting of 0.4 ml root extract, 0.2 ml. 0.1M MnSO_4 , 2 ml. 0.1M citrate (pH 7) or 2 ml. 0.5M pyrophosphate (pH 7) and 0.2 ml. water were made up in test tubes and 0.2 ml. 0.05M H_2O_2 added. The rate of

development and the intensity of the pink or greenish yellow colour was followed for a 15 min period. The strongest reactions were given by extracts from horse radish (*Cochlearia armoracia*) and turnip (*Brassica campestris* L.), with which the colour developed within a minute of adding H_2O_2 . Weaker reactions were given by extracts of carrot peel (*Daucus carota*) and spinach beet (*Beta vulgaris*), dandelion (*Taraxacum officinale*) extracts were inactive. Difficulty was experienced with some extracts, e.g. potato which, owing to the presence of strong direct oxidase systems, rapidly became so dark as to obscure any colour due to manganic complex formation.

Isolation of manganese dioxide

In neutral or acid pyrophosphate, MnO_2 dissolves on the addition of MnSO_4 to give a pink manganopyrophosphate complex (Heintze & Mann, 1946, 1949). At pH values 'much above 8' the complex dismutates into hydrated MnO_2 and manganopyrophosphate (Lingane & Karplus, 1946).



By making use of the dismutation in alkaline solution an attempt was made to prove that the colour formation with root extracts and H_2O_2 was due to the formation of manganic complexes.

To a reaction mixture containing 455 ml. 0.5M pyrophosphate buffer (pH 7), 25 ml. horse radish extract and 20 ml. 0.5M MnSO_4 , H_2O_2 (5 ml., 0.05M) were added at 10 min intervals, with stirring, until the solution became a deep red and further addition of H_2O_2 did not appear to intensify the colour. In all, 25 or 30 ml. of 0.05M H_2O_2 were added, 20 min. after the last addition, 0.15 ml. catalase preparation was added (No H_2O_2 could be detected in the solution at this stage. It was necessary, however, to add the catalase preparation to control (B) and consequently the reaction mixture was similarly treated.) After a further 20 min. the mixture was adjusted to pH 10 (glass electrode) by the addition of 2N NaOH. During the addition of NaOH a brisk current of N_2 was blown through the solution to assist mixing and exclude atmospheric O_2 , thus preventing autoxidation of the remaining MnSO_4 due to local excess of alkali.

The mixture, which was now a dark brown, was allowed to stand for 3 hr. to ensure complete dismutation and then centrifuged. The brown sediment was washed on the centrifuge 4 times with 50 ml. 0.2M pyrophosphate (pH 9.8), 3 times with 50 ml. 0.2M pyrophosphate (pH 7.0) and finally twice with 50 ml. water, to remove Mn^{++} which is absorbed by hydrated MnO_2 . The sediment was ground thoroughly

Table 1. Valency of manganese in oxidation product

(Mn content was estimated colorimetrically, oxidizing capacity by manometric measurement of O_2 output. 0.5 ml suspension, 2.3 ml. N H_2SO_4 in main vessel and 0.2 ml. 0.5M H_2O_2 in 0.1N H_2SO_4 tipped from side arm.)

Preparation no.	Total 0.05M H_2O_2 added in preparation (ml.)	Manganese (mg./ml. of suspension)	Oxygen (μl O_2 /ml of suspension)	Valency	Total MnO_2 * isolated (mg.)	Yield of MnO_2 based on total H_2O_2 added (%)
1	25	0.775	304	3.92	29.5	27
2	30	1.254	518	4.03	51.8	40

* Calculated from oxidizing capacity

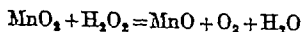
in a mortar and made up in water to 25 ml. Portions were taken for colorimetric determination of manganese and the oxidizing capacity was estimated manometrically with acid H_2O_2 . Two preparations were made: the valency of the manganese was found to be 3.92 in one case and 4.03 in the other (Table 1).

Two control experiments were made, one in which no H_2O_2 was added (A), and the other in which horse radish extract previously heated for 10 min at 100° (B), these mixtures were worked up similarly to the reaction mixture, except that no catalase was added to control (A). Immediately after the addition of catalase to (B), a sample was transferred to a Warburg vessel and the evolution of O_2 was followed. This was complete in 25 min after which 0.2 ml MnO suspension (2.6 mg MnO_2/ml) was tipped from the side arm. No further evolution of O_2 took place showing that the H_2O_2 had been completely decomposed by the catalase. After adjustment to pH 10 and standing for 3 hr the control (B) was yellow brown and control (A) yellow, indicating the presence of a small amount of colloidal MnO . The colour was, however, less brown than the supernatant obtained by centrifuging the reaction mixture at this stage and no sediment was obtained on centrifuging.

It was clear from the experiments that the oxidation depended on the presence of H_2O_2 and a thermolabile factor in the plant extract.

Evidence for the oxidation of manganese based on the accumulation of manganypyrophosphate

It is well known that MnO_2 decomposes H_2O_2 with evolution of O_2 , in water the reaction is catalytic, but in acid solution reduction of MnO_2 may take place and under suitable conditions of acidity (e.g. in 0.1N H_2SO_4) the reaction is stoichiometric,



Experiments were carried out to find out whether the decomposition of H_2O_2 by manganypyrophosphate was catalytic or stoichiometric under the conditions used. The behaviour of hydrated MnO_2 and MnSO_4 under the same conditions was also investigated.

Decomposition of H_2O_2 by MnO_2 and manganypyrophosphate
Known amounts of MnO_2 , or manganypyrophosphate and H_2O_2 were allowed to react and the output of O_2 measured manometrically (Table 2). In water the H_2O_2 was decomposed catalytically by MnO_2 , but in the other media the reaction was generally stoichiometric. In orthophosphate at pH 7 the decomposition was somewhat larger than stoichiometric, but there was also more decomposition in the control. With manganypyrophosphate the reaction was stoichiometric. The spontaneous decomposition of H_2O_2 was slight, and not significantly affected by the presence of $110 \mu\text{g Mn}^{++}$.

Conditions leading to the accumulation of manganypyrophosphate
Since manganypyrophosphate reacts stoichiometrically with H_2O_2 , its accumulation as an oxidation product must depend on its rate of formation being greater than its rate of reduction by H_2O_2 , but during the oxidation of Mn^{++} by horse-radish root extract and H_2O_2 in presence of pyro

phosphate an evolution of oxygen would be expected owing to reduction of part or all of the manganypyrophosphate by the H_2O_2 . If there is no accumulation of manganypyrophosphate and no side reaction the

Table 2 *The decomposition of H_2O_2 by MnO_2 and manganypyrophosphate*

(With MnO_2 the reaction mixtures consisted of $106 \mu\text{g MnO}_2$ (hydrated) and $0.0033\text{M H}_2\text{O}_2$, in 0.033M buffer at pH 7 or in water, with or without the addition of horse radish extract (0.4 ml heated 10 min at 100°). The manganypyrophosphate was performed by the addition of $110 \mu\text{g Mn}^{++}$ to $106 \mu\text{g MnO}_2$ in pyrophosphate at pH 7. The H_2O_2 was tipped from the side arm except when horse radish extract was used when the MnO_2 was tipped. Control experiments were made with $110 \mu\text{g Mn}^{++}$. Theoretical O_2 output for complete catalytic decomposition of H_2O_2 $112 \mu\text{l}$, in absence of catalysis, $24 \pm 4 \mu\text{l}$.)

Time (min)	Oxygen output ($\mu\text{l O}_2$)				
	Water (MnO_2)	Ortho phosphate (MnO_2)	Pyro phosphate (MnO_2)	Horse radish extract heated at 100° (MnO_2)	Manganypyrophosphate
10	104.5	23.0	23.8	25.3	23.5
20	108.5	23.0	24.4	26.0	24.9
60	109.5	29.7	26.1	29.0	27.6
120	111.0	35.3	28.4	29.5	30.3
Control with $110 \mu\text{g Mn}^{++}$					
120	9.0	12.0	3.0	3.0	3.0

total O_2 evolved should be equivalent to the H_2O_2 initially present. If manganypyrophosphate accumulates the total oxygen output will be lower since part

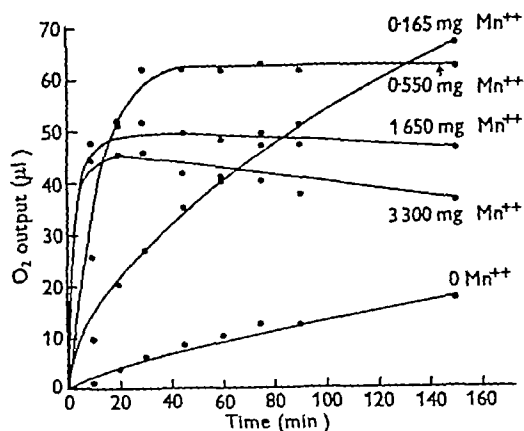


Fig 1 The accumulation of manganypyrophosphate when horse radish extract is treated with H_2O_2 in the presence of added Mn^{++} . The reduction in O_2 output at the higher levels of Mn^{++} indicates manganypyrophosphate accumulation, theoretical O_2 output $112 \mu\text{l}$.

of the H_2O_2 is used up in this accumulation. These reactions can be followed manometrically as shown in Fig 1.

In this experiment the reaction mixtures consisted of 0.4 ml horse radish extract in presence of 0.45 M pyrophosphate at pH 7 and 0.2 ml. of 0.05M H_2O_2 tipped from the side arms, the Mn^{++} added being varied from 0 to 3.3 mg. The slow evolution of O_2 which occurred with the root extract alone was markedly increased on addition of Mn^{++} . The initial output was more rapid with the higher amounts of Mn^{++} , but the total output was greatest with the lowest amount of Mn^{++} . In absence of horse radish extract the output was negligible. These results confirm the view that two reactions are proceeding, the oxidation of the added Mn^{++} by the root extract and H_2O_2 , and the reduction of the oxidation product by H_2O_2 with evolution of O_2 . That the lower total outputs with the higher concentrations of Mn^{++} were due to the accumulation of manganipyrophosphate was supported by the fact that the most intense pink colour was in the flask with the highest concentration of Mn^{++} .

The system may catalyse the oxidation of manganese to the manganic form or to MnO_2 . In orthophosphate at pH 7 a stable manganic complex cannot be formed and breakdown will occur into MnO_2 and MnO , the final product being MnO_2 . If a stable manganic complex can be formed, as in pyrophosphate, it will accumulate provided that the rate of formation is greater than that of reduction by the H_2O_2 . Increasing the concentration of Mn^{++} would therefore favour accumulation by accelerating the rate of oxidation. The fact that Mn^{++} is much less soluble in orthophosphate (pH 7) than in pyrophosphate (pH 7) may partly explain why no accumulation of MnO_2 has been observed in orthophosphate.

Assuming that the primary oxidation product of Mn^{++} is MnO_2 , the following reactions may take place in the pyrophosphate reaction mixture

- (1) $\text{MnO} \rightarrow \text{MnO}_2$,
- (2) $\text{MnO}_2 + \text{H}_2\text{O}_2 \rightarrow \text{MnO} + \text{O}_2 + \text{H}_2\text{O}$,
- (3) $\text{MnO} + \text{MnO}_2 \rightarrow \text{Mn}_2\text{O}_3$,
- (4) $\text{Mn}_2\text{O}_3 + \text{H}_2\text{O}_2 \rightarrow 2\text{MnO} + \text{O}_2 + \text{H}_2\text{O}$

If (2) is more rapid than (1) MnO_2 cannot accumulate, but under conditions where (3) is favoured at the expense of (2), i.e. at high Mn concentration, Mn_2O_3 will be formed, and will accumulate if (4) is slow in comparison with (3).

When hydrated MnO_2 was allowed to react with dilute H_2O_2 in pyrophosphate at pH 7, it was noticed that after the disappearance of the brown MnO_2 the reaction mixture turned pink, and in spite of the presence of excess H_2O_2 this colour faded only slowly. The intensity of this pink colour, which was presumably due to manganipyrophosphate, could be increased if MnSO_4 was added together with the H_2O_2 to the MnO_2 . The formation of manganipyrophosphate in the experiment with horse radish extracts may therefore be due to reaction (3), particularly when large additions of Mn^{++} are made initially.

Since, as is shown below (Fig. 2), reaction (2) is more rapid than (4), the conversion of MnO_2 to manganipyrophosphate favours the accumulation of oxidized Mn.

The reaction between hydrated MnO_2 and H_2O_2 in pyrophosphate at pH 7 is very rapid, particularly at high H_2O_2 concentration, and although that with manganipyrophosphate is slower it is difficult to demonstrate this manometrically.

The conditions finally chosen as satisfactory were as follows: 0.264 mg MnO_2 (hydrated) in 0.16M pyrophosphate at pH 7 in one vessel, and 0.264 mg MnO_2 (hydrated) plus 0.1 ml 0.05M MnSO_4 (to give manganipyrophosphate) in the other vessel. KOH was omitted from the centre cup and the volume of the reaction mixtures was 5 ml. After equilibration, H_2O_2 to give a concentration of 0.0006 M was tipped into both vessels simultaneously by two operators, and the O_2 output was measured at 2 min intervals.

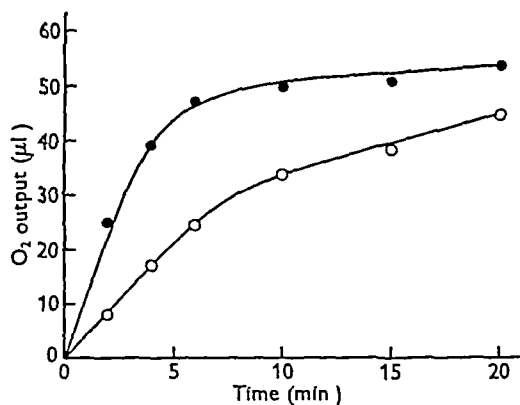
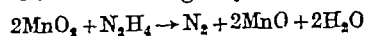


Fig. 2 A comparison of the velocity of the reactions between $\text{MnO}_2 + \text{H}_2\text{O}_2$ and manganipyrophosphate + H_2O_2 , ●—●, $\text{MnO}_2 + \text{H}_2\text{O}_2$, ○—○, manganipyrophosphate + H_2O_2 .

With MnO_2 most of the H_2O_2 was decomposed in the first 5 min with a velocity 2 to 3 times that with manganipyrophosphate (Fig. 2). After 1 hr the output was the same in both cases within experimental error. The comparison is not strictly between MnO_2 and manganipyrophosphate, since manganipyrophosphate would be formed as the reaction between MnO_2 and H_2O_2 proceeded. This probably explains the slow output in the later stages where MnO_2 was used. Furthermore, the hydrated MnO_2 was in the form of a suspension and the rate of the reaction would depend on the particle size, whereas the manganipyrophosphate was in solution.

Estimation of manganipyrophosphate by hydrazine

It is known that MnO_2 reacts quantitatively with hydrazine in the following way



Manganipyrophosphate also reacts with hydrazine giving a gas output slightly higher than that required by theory (Table 3), and can be estimated in this way. Experiments in which the horse radish extract was allowed to react with H_2O_2 in 0.67M pyrophosphate and in the presence of varying concentrations of MnSO_4 were carried out manometrically in an atmosphere of N_2 . When the O_2 output ceased, hydrazine was tipped from the side arm, and a rapid evolution of gas, presumably N_2 ,

occurred. The output was greatest where the highest accumulation of manganypyrophosphate was expected and was accompanied by discharge of the pink colour. If the reactions in the first part of the experiment led only to the evolution of O_2 and the accumulation of manganypyrophosphate, then the combined O_2 and N_2 gas outputs should be equivalent to the H_2O_2 added. In experiments using 0.4 ml horse radish extract, the combined gas outputs were considerably lower than this, but were only 5–10% lower when 0.1 ml extract was used (Table 4). The reduction in the amount of extract used led to a decrease in the accumulation of manganypyrophosphate. The fact that the output of N_2 was less than the theoretical suggests either that part of the H_2O_2 was lost in a side reaction, or that part of the MnO_2 or manganypyrophosphate formed was reduced by the extract. Reduction of MnO_2 , and to a less extent of manganypyrophosphate, takes place when these compounds are incubated with the horse radish extract (Table 3).

The properties of the manganese oxidizing system

It has been demonstrated in the previous experiments that horse radish extracts in presence of H_2O_2 can bring about the oxidation of Mn^{++} . To identify and estimate the oxidation product it was necessary to carry out the experiments under conditions in which a stable manganic complex could accumulate, i.e. in pyrophosphate at pH 7 and with a high concentration of Mn^{++} . If the system is of physiological significance, however, oxidation should take place at low concentration of Mn^{++} . The oxidation product does not accumulate under these conditions because it reacts with H_2O_2 with evolution of O_2 as rapidly as it is formed. The oxidation can therefore be demonstrated manometrically.

The effect of small amounts of Mn^{++} on the decomposition of H_2O_2 by horse radish extract. With most of the horse radish root extracts, but not with other root extracts, an increase in the rate of decomposition of H_2O_2 occurred on addition of very small amounts of $MnSO_4$. The experiments were carried

Table 3 *Reaction of MnO_2 and manganypyrophosphate with hydrazine in presence and absence of horse radish extract*

(The MnO_2 content of a suspension of hydrated MnO_2 was determined by oxalate titration. 0.4 ml portions (0.984 mg MnO_2) were suspended in 0.67M pyrophosphate buffer in Warburg vessels. The gas space was then filled with N_2 . After equilibration 0.2 ml. saturated aqueous N_2H_4 . H_2SO_4 was tipped from the side arm, the gas output was measured. Similar experiments were carried out with manganypyrophosphate formed by adding 0.2 ml. of 0.1M $MnSO_4$ to the reaction mixtures. Experiments were also made to determine the effect of additions of 0.4 ml horse radish extract on the estimation.)

Method of estimation	Reaction mixture	Gas output (μ l)	MnO_2 (mg)	Recovery (%)
Oxalate titration	MnO_2	—	0.984	—
Acid H_2O_2 (manometric)	MnO_2	248.5	0.965	98.0
N_2H_4 (manometric)	MnO_2	128.0	0.994	101.0
	MnO_2 tipped into extract + N_2H_4	125	0.972	98.5
	MnO_2 + extract incubated 30 min	116.0	0.901	91.5
	MnO_2 + extract incubated 60 min	110.8	0.861	87.5
	Manganypyrophosphate	133.5	1.037	105.0
	Manganypyrophosphate + extract incubated 30 min	131.0	1.02	103.5
	Manganypyrophosphate + extract incubated 60 min	127.0	0.987	100.5

Table 4 *Accumulation of manganypyrophosphate*

(Reaction mixtures consisted of horse radish extract and $MnSO_4$ as below in 0.67M pyrophosphate at pH 7, 0.2 ml 0.05M H_2O_2 was tipped from the side arm and the O_2 output was measured. When the O_2 output stopped 0.2 ml. saturated aqueous N_2H_4 . H_2SO_4 was tipped from the second side arm and the N_2 output measured. Gas space N_2 .)

	Extract A						Extract B (0.2 ml.)					
	(0.4 ml.)			(0.1 ml.)			0.55	1.375	2.75	5.50	8.25	
$MnSO_4$ added (mg Mn)	0.55	1.65	2.75	1.65	2.75		0.55	1.375	2.75	5.50	8.25	
(1) O_2 output (μ l)	66.2	49.0	49.5	85.8	79.3		76.2	75.6	71.0	62.5	55.3	
(2) N_2 output (μ l)*	25.1	43.6	48.5	25.7	27.9		22.9	30.2	36.2	41.8	46.8	
(3) $O + N_2$ output (μ l.)	91.3	92.6	98.0	111.5	107.2		99.1	105.8	107.2	104.3	100.3	
(4) Theory for $O_2 + N_2$ output (μ l)†	116.0	116.0	116.0	116.0	116.0		112.8	112.8	112.8	112.8	112.8	

* Uncorrected for 105% recovery (see Table 3)

† Half O_2 output obtained by tipping the H_2O_2 into excess hydrated MnO_2 in N H_2SO_4

out manometrically with 0.4 ml root extract in presence and absence of 0.033M ortho or pyro phosphate buffer, 0.2 ml of 0.05M H_2O_2 being tipped from the side arm after equilibration. The results of a set of experiments in the absence of buffer are given in Fig. 3. In absence of added Mn^{++} a slow decomposition of H_2O_2 occurred. This was in general increased by the addition of $2.2 \mu\text{g}$ Mn^{++} , and increased progressively with increasing amounts of Mn^{++} . In general, the experiments were carried out in air, but similar results were obtained when the gas space was filled with N_2 , showing that direct oxidases were not concerned in the reaction.

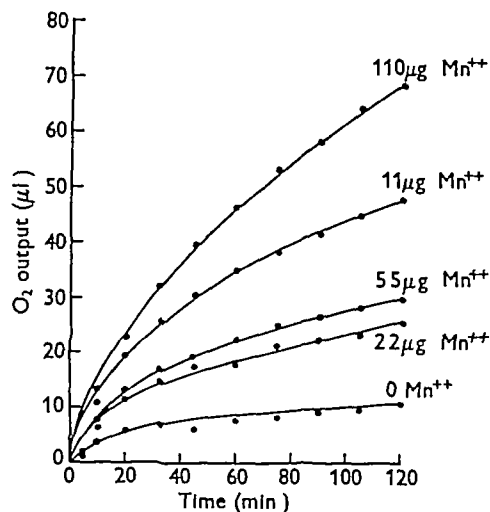


Fig. 3 The effect of added Mn^{++} on the rate of decomposition of H_2O_2 by horse radish extract

In ortho and pyro phosphate at pH 7 the rate of decomposition of H_2O_2 by the extract was higher than in the unbuffered extract (pH 4-5), but a similar effect of Mn^{++} was found. In orthophosphate at pH 7 with $11 \mu\text{g}$ Mn^{++} added the output stopped in 80 min, the total output was $70 \mu\text{l}$ O_2 , about 63% of that required for complete decomposition of the H_2O_2 added ($112 \mu\text{l}$ O_2). No residual H_2O_2 could be detected in the reaction mixture and some H_2O_2 must therefore be lost in side reactions. In orthophosphate at pH 5 the rate of decomposition of H_2O_2 , both in presence and absence of added Mn^{++} , was less than at pH 7. In general 0.4 ml of extract produced the effects described above, with smaller amounts the response to added Mn^{++} was less, and usually with 0.05 ml of extract little or no increase in the rate of decomposition of H_2O_2 was observed with additions of $110 \mu\text{g}$ Mn^{++} . Some extracts gave little response to additions of $11 \mu\text{g}$ Mn^{++} even when 0.4 ml was used.

Control experiments (Table 3) showed that in all the media used the decomposition of H_2O_2 in absence of extract was not appreciably affected by addition of Mn^{++} and was not sufficiently great to affect the results of the above experiments.

The rate of decomposition of H_2O_2 by the horse radish extract in different media with and without added Mn^{++} was reduced by previous heating at 100° . The effect of heat was somewhat variable and even after heating for 10 min a slight activity remained in some cases.

It has already been shown that when large amounts of Mn^{++} are added to horse radish extract and H_2O_2

the manganese is oxidized and the oxidation product reacts with H_2O_2 with evolution of O_2 . It is considered therefore that the increase in rate of decomposition of H_2O_2 with small amounts of Mn^{++} is likewise due to oxidation of manganese. Since under the conditions of the experiments the reaction of MnO_2 and manganopyrophosphate with H_2O_2 is stoichiometric (Table 2) it is obvious that if small amounts of Mn^{++} cause a large increase in O_2 evolution the Mn^{++} must undergo a cycle of oxidation and reduction.

The effect of various metals on the decomposition of H_2O_2 by horse-radish extract. The effect of Mn^{++} , Co^{++} , Cu^{++} , Zn^{++} , Ni^{++} , Fe^{++} and Fe^{+++} on the decomposition of H_2O_2 by the horse radish extract was tested. At the concentration used ($22 \mu\text{g}/3 \text{ ml}$) only Mn^{++} produced an increase in the rate of decomposition.

Effect of variation in H_2O_2 concentration. The effect of varying the H_2O_2 concentration on the decomposition of H_2O_2 by horse radish extract with $22 \mu\text{g}$ added Mn^{++} in 0.033M orthophosphate at pH 7 was tested. The system was active over the range 0.02-0.00083M- H_2O_2 . With the higher concentrations an inhibitory effect was apparent in time. There was no reduction in the initial velocity, even with the lowest concentration.

Activation by an ether soluble factor. The response of relatively inactive extracts, previously mentioned

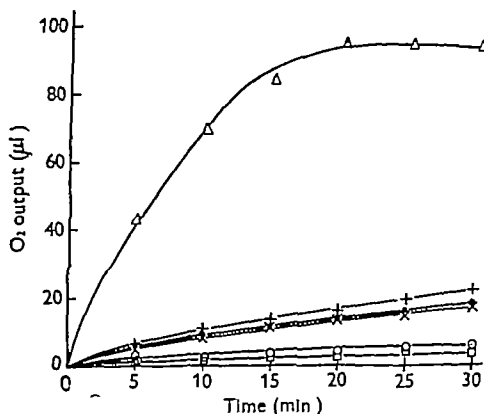


Fig. 4 The activation of horse radish extract + Mn^{++} by an ether soluble factor in the extract, $\bullet-\bullet$, extract, $+-+$, extract + $55 \mu\text{g}$ Mn^{++} , $\triangle-\triangle$, extract + ether soluble factor + $55 \mu\text{g}$ Mn^{++} , $\times-\times$, extract + other soluble factor, $\square-\square$, ether soluble factor + $55 \mu\text{g}$ Mn^{++} , $\circ-\circ$, $55 \mu\text{g}$ Mn^{++}

(p. 260), was increased considerably by the addition of an ether extract of acidified active horse radish extract. The extract used was made from a residue from a peroxidase preparation and had previously been saturated with $(\text{NH}_4)_2\text{SO}_4$ and filtered, 200 ml was acidified to pH 3 with $\text{N H}_2\text{SO}_4$ and shaken

3 times with 30 ml ether, the combined ether extracts were taken to dryness at room temperature and the residue suspended in 5 ml water. The effect of 0.5 ml of this suspension on the rate of decomposition of H_2O_2 by horse radish extract which gave little response to $55 \mu\text{g Mn}^{++}$ was tested in 0.033 M-orthophosphate at pH 7 in the presence and absence of Mn^{++} (Fig. 4). While the ether soluble factor or Mn^{++} alone caused little or no increase in the activity of the extract, together they gave a marked increase. The ether soluble factor was unaffected by heating for 15 min in a boiling water bath, but was destroyed by ashing. It was clear therefore that a thermostable factor was a component of the manganese oxidation system.

The oxidation of manganese by a peroxidase system

The fact that H_2O_2 was necessary for the oxidation suggested that the thermolabile factor was a peroxidase. It was found that, on precipitation with $(\text{NH}_4)_2\text{SO}_4$ and dialysis, peroxidase preparations, obtained by the method of Keilin & Mann (1937), gave no evidence of manganese oxidation by colorimetric tests. Positive tests, however, were obtained with these and more highly purified peroxidase preparations, when the ether soluble factor, previously described, or certain peroxidase substrates, such as *p*-cresol, were added. It was noted that whereas in the absence of manganese, *p*-cresol was oxidized by the peroxidase and H_2O_2 to give an insoluble white oxidation product, in the presence of Mn^{++} the solution remained clear and the coloured manganic complex appeared. These results suggested that the ether soluble factor of the horse radish extracts is a substrate for peroxidase and that manganese reduces the oxidized substrate and is thereby itself oxidized.

The oxidation of manganese by systems containing peroxidase can be followed by manometric measurement of the O_2 evolved. In the experiments shown in Fig. 5 the oxidizing system was in the main vessel, 7 μg of a peroxidase preparation with P.Z. 290, 0.00033 M *p*-cresol in 0.033 M orthophosphate at pH 7 to which varying additions (5–33 μg) of Mn^{++} were made, 0.2 ml 0.05 M H_2O_2 was tipped from the side arm after equilibration. In absence of added Mn^{++} no evolution of oxygen took place, but with increasing amounts of Mn^{++} increases in the velocity and in the total output were observed. The theoretical output (112 $\mu\text{l O}_2$) was obtained with 33 μg added Mn^{++} .

In the reaction mixture without Mn^{++} , a white precipitate was present, indicating that the *p*-cresol had been oxidized. This precipitate was smaller in the vessels with 5–11 $\mu\text{g Mn}^{++}$ and was not visible with 22–33 $\mu\text{g Mn}^{++}$. No significant decomposition of H_2O_2 was observed in control experiments. These results are in agreement with the conclusion previously reached that manganese reduces an oxidation product of *p*-cresol and is itself oxidized. In the

absence of sufficient Mn^{++} the oxidation product accumulates. In orthophosphate at pH 7 the oxidized manganese reacts stoichiometrically with H_2O_2 . In presence of sufficient Mn^{++} , therefore, accumulation of the oxidation product does not occur and the H_2O_2 is completely decomposed.

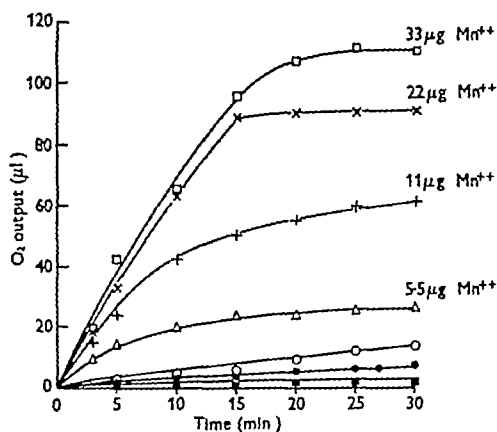


Fig. 5 The decomposition of H_2O_2 by the system peroxidase, *p*-cresol and Mn^{++} . \triangle — \triangle , +—+, \times — \times , \square — \square , peroxidase + *p*-cresol with varying Mn^{++} additions, \odot — \odot , peroxidase heated 10 min at 100° + *p*-cresol + 22 $\mu\text{g Mn}^{++}$, \bullet — \bullet , peroxidase + 22 $\mu\text{g Mn}^{++}$ or *p*-cresol + 22 $\mu\text{g Mn}^{++}$, \blacksquare — \blacksquare , peroxidase + *p*-cresol, buffer, orthophosphate.

DISCUSSION

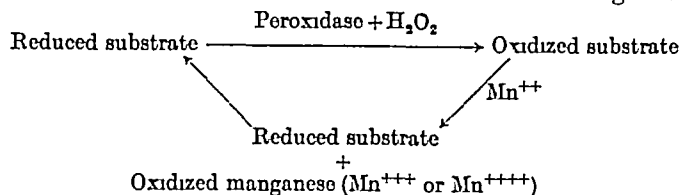
The results obtained provide conclusive evidence that a system is present in horse radish extract which in the presence of H_2O_2 oxidizes manganese. Colour tests with other root extracts suggest that this reaction is a general phenomenon. The valency of the manganese oxidation product has not been established, but under conditions where manganic manganese is stable the product accumulates in this form. This may arise by a reaction between MnO and MnO_2 .

In addition to H_2O_2 , a thermolabile and a thermostable factor, which are probably peroxidase and a peroxidase substrate respectively, are necessary for the oxidation. The thermolabile properties of the natural system agree with those attributed to peroxidase by Gallagher (1924) and Herrlinger & Kiermeier (1944), in that prolonged heating at 100° is necessary for complete inactivation. Also, of the root extracts tested those such as horse radish and turnip, which are good sources of peroxidase, appear to be the most active and the maximum activity as shown by the decomposition of H_2O_2 is reached at low H_2O_2 concentrations.

On the basis of the results obtained with the partially purified peroxidase preparations the working hypothesis is advanced that manganese oxidation is brought about by the oxidized substrate

of the peroxidase, which is involved in a cycle of oxidation and reduction

The results of the present work suggest a connexion between manganese and plant peroxidase



It is necessary to postulate such a cycle since only catalytic amounts of substrate are required experimentally. Preliminary experiments suggest that not all peroxidase substrates are active in the cycle. Thus manganese oxidation has been obtained with the system using *o* and *p* cresol but not with pyrogallol. The experiments with *p* cresol indicate that it is an intermediate and not the final oxidation product which brings about the oxidation of manganese.

Comparison of the activities of the horse radish extracts, used with that of the peroxidase preparation in presence of *p* cresol, suggests that the activity of the extracts is limited by the amount of the peroxidase substrate present. In agreement with this, activation of comparatively inactive horse-radish extracts could be brought about by the addition of an ether soluble factor from active extracts.

At the concentrations of H_2O_2 used experimentally part or all of the manganese oxidation product is reduced by the H_2O_2 . This involves the Mn^{++} as well as the peroxidase substrate in a cycle of oxidation and reduction. In manometric experiments with small amounts of added Mn^{++} this results in decomposition of the H_2O_2 with evolution of O_2 . These experiments were carried out at concentrations of H_2O_2 relatively high compared with those that would be present *in vivo*. At very low concentrations of H_2O_2 it appears probable that the oxidized Mn would react preferentially with metabolites other than H_2O_2 . This would lead to a transfer of oxygen from H_2O_2 to plant metabolites without evolution of O_2 . Such a transfer would involve the manganese in a cycle of oxidation and reduction and the hypothesis is put forward that this cycle may be responsible, at least in part, for the effect of manganese on plant respiration shown by Lundegårdh (1939). At very low concentrations of H_2O_2 , if oxidation is rapid compared with reduction, the oxidation product would accumulate. It is possible that such conditions exist in soils where microbiological oxidation of Mn^{++} leads to an accumulation of manganese higher oxides. It has been suggested that the underlying factor of Mn-Fe antagonism in the growth of plants is the oxidation of ferrous iron to ferric by manganese (Hopkins 1930, Shive 1941). Such a mechanism depends on the presence in the plant of a system oxidizing manganese.

If manganese can also be oxidized through the agency of the peroxidase like activity of haem or haematin derivatives, or by milk peroxidase, it is possible that manganese oxidation may occur in animal tissues.

It is known that H_2O_2 is formed in several oxidation reactions catalysed by enzymes, e.g. xanthine oxidase, amino acid oxidase and glucose oxidase. Such H_2O_2 can be used in promoting secondary or coupled oxidations, thus Thurlow (1925) and Harrison & Thurlow (1926) used an enzyme system to provide H_2O_2 for the peroxidase system and Keilin & Hartree (1936, 1945) similarly demonstrated oxidation of alcohols to aldehydes by means of catalase together with H_2O_2 formed by the previously mentioned enzyme systems.

In preliminary experiments colorimetric evidence has been obtained that H_2O_2 formed by the xanthine oxidase—hypoxanthine system may be used to bring about the oxidation of manganese by the horse radish extract. It seems possible that an enzyme system producing H_2O_2 provides a suitable means of maintaining a low concentration of H_2O_2 and would facilitate the study of the role of the oxidized Mn in plant respiration. Further work along these lines is in progress.

Lastly, it should be pointed out that the system may be able to oxidize metallic ions other than Mn^{++} . It has been possible to follow the oxidation of manganese manometrically, as the oxidation product decomposes H_2O_2 with evolution of O_2 . The fact that the other metallic ions so far tested have not shown such activity is no proof that they are not oxidized by the system.

SUMMARY

1 A system which oxidizes manganese in the presence of hydrogen peroxide has been demonstrated in horse radish root extracts. This system also exists in other root extracts.

2 Under suitable conditions (i.e. in pyrophosphate or citrate at pH 7) the oxidation product accumulates as a coloured manganic complex. Using horse radish root extract, manganese dioxide was isolated by the dismutation of manganopyrophosphate at weakly alkaline reaction. The oxidation product decomposes hydrazine and can be estimated manometrically by means of this reaction.

3 An increase in the rate of decomposition of hydrogen peroxide was observed on adding small amounts of manganous sulphate to horse radish extracts. This effect was attributed to manganese oxidation.

4 The manganese oxidizing system in horse-radish extract consists of a thermolabile and a thermostable factor together with hydrogen peroxide. Partially purified peroxidase preparations, in the presence of certain peroxidase substrates and hydrogen peroxide, oxidize Mn^{++} . It is suggested that the thermolabile and thermostable factors in

the extract are peroxidase and peroxidase substrate respectively.

5 The hypothesis is advanced that the manganese reduces the oxidized peroxidase substrate and thereby is itself oxidized. This involves the substrate in a cycle of oxidation and reduction. At low hydrogen peroxide concentrations, the manganese oxidation product may react with plant metabolites other than hydrogen peroxide, thus would involve the manganese in an oxidation-reduction cycle, which may explain its effect on plant respiration.

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The Biochemistry of Locusts

1 THE CAROTENOIDS OF THE INTEGUMENT OF TWO LOCUST SPECIES (*LOCUSTA MIGRATORIA MIGRATORIOIDES* R. & F. AND *SCHISTOCERCA GREGARIA* FORSK.)

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(Received 22 February 1949)

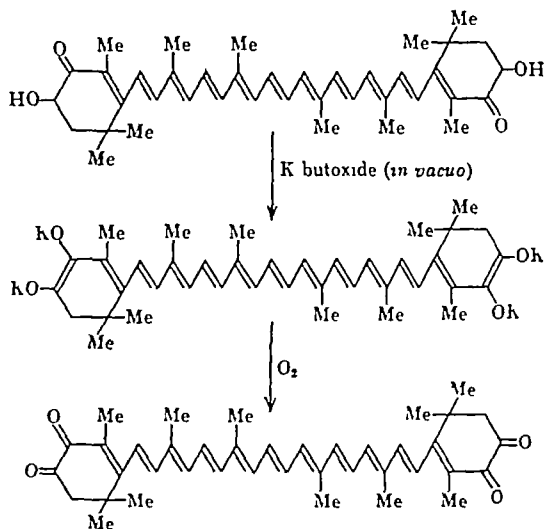
A research programme on the biochemistry of locust pigmentation, with special reference to the problem of swarming, has recently been initiated. An essential preliminary to the study of carotenoid metabolism in these insects (Goodwin, 1949) was the identification of the pigments concerned. Lederer (1935), quoting unpublished work carried out by Volkonsky and himself, reported the presence of a mixture of xanthophylls and carotene in adult *Schistocerca gregaria* Forsk. Chauvin (1941) noted an unidentified pink pigment in hoppers and a mixture of α and β carotenes in adult insects of the same species. No data on *Locusta* species have been recorded.

We have found that hoppers and adult insects of both *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* yielded, on extraction with acetone after removal of the alimentary tract, a mixture of two carotenoids. These pigments have been identified as β carotene and astaxanthin. Small amounts of lutein (xanthophyll) and chlorophyll were occasionally encountered, but they probably originated in small amounts of unremoved alimentary tract or in small pieces of grass which can become tightly lodged in the buccal cavity and the claws of the forelegs.

The pigments were identified by their chromatographic and spectrographic properties, insufficient

material was available to make crystallization feasible. The most rigid chromatographic criterion applied was that of the mixed chromatogram. When the suspected β carotene or astaxanthin fraction was admixed with an authentic sample of the corresponding pigment (crystalline β carotene or astaxanthin isolated from hypodermis of lobster (*Homarus vulgaris* H. M. Edwards) and prawn (*Nephrops norvegicus* L.), and the mixture chromatographed on an appropriate adsorbent, no separation was obtained. The absorption spectra of the purified fractions were compared with those of the authentic pigments over the range 550–400 $m\mu$ in a variety of solvents, in all cases the spectra were almost exactly superposable. In investigations in the carotenoid series, in which the absorption maximum of different components can differ only slightly, it should be emphasized that it is only when the complete absorption curves obtained in different solvents are compared that identification becomes unequivocal, mere recording of maxima is often insufficient.

Astaxanthin was further characterized by its reaction with potassium butoxide *in vacuo* to form a bluish purple enolic salt which on the admission of air is almost instantaneously converted into the orange coloured astacin (3,4,3',4'-tetraketo- β -carotene, Kuhn & Sørensen, 1938). Further, treatment of the locust astaxanthin with alkali yielded a product which was chromatographically and spectroscopically identical with astacin produced in a similar way from lobster astaxanthin (Kuhn & Lederer, 1933). Almost all the astaxanthin isolated from locusts was unesterified.



A large number of eyes and wings of *Locusta* and *Schistocerca* were examined separately for carotenoids. All were found to contain β carotene and free (unesterified) astaxanthin. The astaxanthin in

the wings occurs in combination with a protein, but in all probability β carotene does not.

Examination of the fatty tissues, blood and eggs of *Schistocerca* and *Locusta*, revealed the presence of β carotene only, neither α carotene nor any xanthophylls could be detected.

Finally, advantage was taken of the availability of a large number of live locusts to determine whether or not they produce vitamin A. Using the most rigid tests for detecting vitamin A, it was never possible to demonstrate the presence of the vitamin in either species.

EXPERIMENTAL

Extraction, separation and identification of the carotenoids of Locusta and Schistocerca

The locusts were killed by cutting off the head just behind the pronotum, the thorax and abdomen were then opened ventrally and the fatty tissues and internal organs removed and discarded. The remaining tissues were thoroughly ground up with acid washed silver sand and a small amount of anhydrous Na_2SO_4 and extracted 3–4 times with acetone. The combined acetone extracts were concentrated at the pump at a temperature not exceeding 35°, the final traces of acetone were removed at a slightly higher temperature in a stream of N_2 . The residue was then dissolved in light petroleum (b.p. 40–60°) and chromatographed using defatted bone meal as adsorbent (Mann, 1943, Goodwin & Morton, 1946, Glover, Goodwin & Morton, 1947). Elution with light petroleum removed the hydrocarbon carotenoids (fraction A) and the xanthophylls were subsequently removed from the column with acetone (fraction B).

Fraction A was found to have an absorption spectrum identical with that of β carotene. Its identity with this pigment was confirmed by admixing a portion with a solution containing an approximately equal amount of crystalline β carotene and chromatographing on activated alumina (Grade O, Peter Spence and Co.), even on slow development down a long column (20–30 cm) no separation into two fractions could be obtained.

Fraction B was examined in one of three ways.

(a) The fraction was dissolved in light petroleum and partitioned between this solvent and 90% (v/v) aqueous methanol. The epiphase contained very small amounts of a yellow pigment whilst the main fraction was hypophasic and coloured orange red.

The epiphasic layer was examined spectroscopically and chromatographically, from this examination the presence of small amounts of lutein (xanthophyll) esters was indicated. The identification was not pursued further.

The orange red methanol solution was diluted with 3 vol of water and the pigment extracted with light petroleum. The extract, when washed free from acetone with water and dried over anhydrous Na_2SO_4 , was chromatographed either on a column of deactivated alumina or CaCO_3 . (Deactivated alumina was prepared by mixing activated alumina (Peter Spence and Co.) with 10% of its weight of methanol, allowing to stand for 1 hr, filtering the methanol off and drying at room temperature.) Using either adsorbent the pigment was adsorbed as a red zone at the top of the column, elution with ethanol removed a very small amount of brown material which was probably esterified astaxanthin. The

main pigment was eluted with a 2% (v/v) solution of glacial acetic acid in ethanol, this is a characteristic property of astaxanthin (Kuhn & Sørensen, 1938). A very small amount of pink material, which remained on the column, could be eluted with ethanol containing 2.5% (w/v) of KOH, thus, according to Kuhn & Sørensen, is a characteristic property of astacin, and its presence in our extracts was probably caused by oxidation of astaxanthin during the working up process, for astacin does not occur in nature (Kuhn, Stene & Sørensen, 1939).

The glacial acetic acid solution of astaxanthin was diluted with an equal volume of water, and the pigment extracted with ether, this extract was then identified as described below.

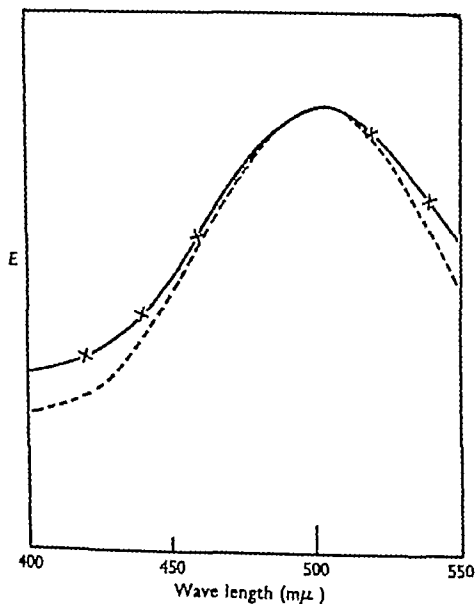


Fig 1 The absorption spectra of astaxanthin obtained from locusts (—) and from lobsters (—x—x—x—), solvent, CS₂. Scales for the extinctions (*E*) have been chosen so that the ordinates representing *E*_{max} for the two samples are equal.

(b) The extract was dissolved in light petroleum and chromatographed directly, using deactivated alumina as adsorbent. Small amounts of lutein (xanthophyll) were eluted with light petroleum containing 4% (v/v) of acetone, the astaxanthin was then separated and eluted as described under (a) above.

(c) The light petroleum solution was extracted with a 1% (w/v) solution of Na₂CO₃. This removed astaxanthin from the petroleum phase and at the same time converted it into astacin (Kuhn & Sørensen, 1938). The Na₂CO₃ solution was acidified with acetic acid and the astacin extracted with ether. After removing the ether, the residue was dissolved in light petroleum containing a small amount of ether and chromatographed on deactivated alumina. The pigment was purified by eluting with ethanol containing 2.5% (w/v) of KOH.

The pigment isolated by procedures (a) and (b) was considered to be astaxanthin and that by procedure (c) astacin produced from astaxanthin. Further proof of their identity

was obtained (i) by comparing their absorption spectra with those of authentic samples of astaxanthin and astacin obtained from marine Crustacea (Figs 1 and 2), (ii) by failure to separate chromatographically a mixture of 'locust' and 'lobster' astaxanthin or astacin into two components, and (iii) by the conversion of astaxanthin into a purple potassium (enol) salt which was rapidly oxidized to astacin in the presence of O₂. To prepare the purple K salt a solution of the pigment in light petroleum was placed in a filter flask closed with a rubber bung from which was suspended by thread a small test tube containing K butoxide. The flask was evacuated to about 1 mm Hg, the light petroleum was evaporated and the pigment remained as a thin smear on the

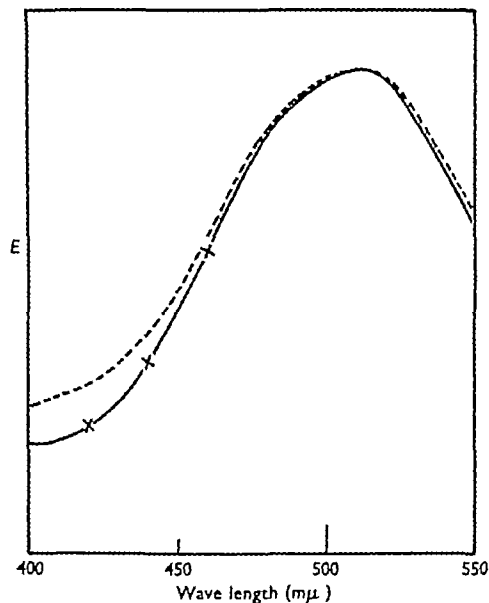


Fig 2 The absorption spectra of astacin obtained from locusts (—) and from lobsters (—x—x—x—), solvent, CS₂. Scales for the extinctions (*E*) have been chosen so that the ordinates representing *E*_{max} for the two samples are equal.

bottom of the flask. The flask was then tilted so that the K butoxide poured on to the pigment, immediately a deep purple coloration was produced. On admitting air this immediately turned orange, this orange pigment possessed all the chromatographic and spectroscopic properties of astacin prepared from lobster astaxanthin.

When free and esterified astaxanthin are partitioned between light petroleum and 90% (v/v) aqueous methanol the free form accumulates in the methanol layer (hypophase) and the esterified pigment in the light petroleum layer (epiphase, Kuhn, Lederer & Deutsch, 1933). All the astaxanthin fractions isolated in this investigation were almost completely hypophase in this test, indicating that the free pigment predominated.

When locust eyes were examined they were treated in the same way as the integuments, the wings, however, were subjected to different treatment.

Examination of locust wings Various samples of wings from *Locusta*, *Schistocerca gregaria* and one sample of wings

of red locust (*Nomadacris septemfasciata* Serville) were examined in the following way. The wings were placed in a Waring mixer and extracted for 10 min with light petroleum (b.p. 40–60°), the light petroleum was filtered at the pump through a G 4 sintered glass filter and examined chromatographically and spectroscopically. The fraction contained only one pigment, β carotene. The wing residue was divided into two portions, one of which was extracted with acetone in the Waring mixer. The acetone fraction was dark orange in colour and when tested was shown to contain only unesterified astaxanthin. This was good presumptive evidence that the astaxanthin was held attached to a protein.

Consequently, the second portion of the wings was extracted with about 150 ml water containing two drops of NH_3 solution (sp. gr. 0.88) in the Waring mixer. The resulting mush was centrifuged and the dark brown supernatant liquid decanted off. Addition of solid $(\text{NH}_4)_2\text{SO}_4$ to saturation threw down a brown protein, leaving the supernatant liquid a pale yellow colour. The protein was centrifuged off and redispersed in a small volume of water. Shaking this solution with light petroleum extracted a small amount of β carotene. After the protein pigment complex was broken down by the addition of an equal volume of ethanol the pigment was easily extractable with light petroleum. It showed all the properties of free astaxanthin. The wing tissues remaining after extraction with water were extracted with acetone, a small amount of astaxanthin was obtained, probably owing to the presence of unextracted protein. It is important to note that in similar tests with locust integuments it has not yet been possible to detect such an astaxanthin complex.

Body fat and eggs Samples of body fat and eggs were ground up with anhydrous Na_2SO_4 and the pigment extracted with acetone. On transferring the pigment to light petroleum and chromatographing on activated alumina only one pigment was detected. Spectroscopic data and a 'mixed' chromatogram indicated that the pigment was β carotene.

Extraction of astaxanthin from lobsters It was necessary to obtain authentic specimens of astaxanthin with which to compare the locust pigment, lobsters were considered the best source. The methods of extraction are described in a subsequent communication (Goodwin & Srisukh, 1949).

Blood About 1 ml of blood was obtained from about fifty immature adult *Locusta*. It was examined according to the method of Glover *et al.* (1947). The only pigment obtainable was β carotene which was identified in the usual way. The concentration of β carotene in the blood was approx. 600 $\mu\text{g}/100\text{ ml}$. A similar result was obtained with *Schistocerca* blood, but the β carotene content was considerably higher, approx. 3000 $\mu\text{g}/100\text{ ml}$.

Tests for the presence of vitamin A in Locusta and Schistocerca

A number of acetone extracts of whole *Locusta* and *Schistocerca* were prepared as previously described. After removal of the acetone the extracts were dissolved in light petroleum and chromatographed on defatted bone meal according to the method of Glover *et al.* (1947). Two fractions were obtained, one of which was eluted with light petroleum and which should have contained any esterified vitamin A, and one which was eluted with acetone and should have contained any free vitamin A. The solvents were removed from the two fractions and the residues made up in cyclohexane and examined in two ways: (a) the absorption

spectrum of the colour produced by reaction of the extract with a CHCl_3 solution of SbCl_5 was examined in the Hilger Nutting visual spectrophotometer to determine the wavelength of the absorption band, and in the Beckman quartz photoelectric spectrophotometer for the measurement of the extinction at 617 $\text{m}\mu$ (i.e. at the λ_{max} for the SbCl_5 vitamin A colour test). In no case was an absorption band noted at 617 $\text{m}\mu$, nor was any absorption of a transitory nature observed at 617 $\text{m}\mu$, in fact, it always increased considerably on standing. Thus, using the SbCl_5 test, no positive evidence of the presence of vitamin A, either in the free or esterified form, could be obtained.

(b) The direct absorption spectra of the cyclohexane solutions were recorded using the Beckman instrument. Considerable absorption existed at 328 $\text{m}\mu$, but when corrected for absorption not due to vitamin A, most of the curves were 'uncorrectable', that is, in most cases the absorption calculated as due to vitamin A possessed a small negative value. Thus again no evidence for the presence of vitamin A could be obtained.

DISCUSSION

The present investigation has demonstrated the presence of β carotene and astaxanthin in two species of locusts, *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk., β carotene exists in the integument, wings, fatty tissues, eggs and eyes, and astaxanthin in the integument, wings and eyes.

Almost all the astaxanthin occurring in the integument is unesterified, it has not yet been possible to demonstrate, as in the case of the wings and lobster cuticle (Kuhn & Lederer, 1933), the presence of astaxanthin as a protein complex in the locust integument.

It is hoped to present in a future paper data concerning the quantitative distribution of β carotene and astaxanthin in locusts during the various stages of development. As this survey will not include separate data on eyes and wings it is interesting to note here that the mean concentrations in the eyes and wings are, respectively, 13.9 and 5.1 $\mu\text{g}/\text{g}$ of β carotene and 7.45 and 10.4 $\mu\text{g}/\text{g}$ of astaxanthin. Both the flying wings and the elytra have the same concentration of astaxanthin. Whilst the relative amounts of the two pigments in the eyes are very similar to those found in the integument of adults (Goodwin, 1949) astaxanthin tends to preponderate in the wings. From the figures just quoted it can be calculated that one pair of locust eyes contain 0.130 μg of β carotene and 0.048 μg of astaxanthin, the corresponding values for one set of wings are 0.0255 and 0.356 μg .

The wing astaxanthin occurs in combination with a protein, but in all probability β carotene does not, for when the ground tissue is extracted with light petroleum (b.p. 40–60°), a solvent which does not liberate carotenoids from their protein complexes, β carotene, but not astaxanthin, is extracted. When

the residue is extracted in the water containing about 0.05% of ammonia (sp gr 0.88) a brown solution is obtained from which a brown protein is precipitated at full saturation with ammonium sulphate. When the brown protein is dissolved in water and denatured by the addition of ethanol, astaxanthin, but not β carotene, appears in a light petroleum extract. The attachment of astaxanthin to a protein of the wings of the red locust, *Nomadacris septemfasciata* Serville, has also been demonstrated. All these results confirm and extend the claims of Junge (1941) and Okay (1947) that a component of the colour complex of insects, in particular the wings, is an (unspecified) caroteno protein, that Okay noted such a pigment in the acridid, *Acrida turturita* L., is of special interest. More recently, and following our preliminary notice of the presence of astaxanthin in locusts (Goodwin & Srisukh, 1948), Okay (1948) has stated that the carotenoid of the protein complex in a number of insects is astaxanthin.

The demonstration of the presence of β carotene only in the fatty tissues and eggs of *Locusta* and *Schistocerca* confirms in general the previous statement of Chauvin (1941) that β carotene exists in the eggs of *Schistocerca*, however, he also claimed to have detected small amounts of α carotene. It is interesting to note that investigations on the body fat and gonads of another acridid, the grasshopper (*Melanoplus bivittatus* Say), have produced similar results (Grayson, 1942, Grayson & Tauber, 1942).

The carotenoids in locust blood are interesting in two respects. (1) The carotene levels are extremely high compared with those usually obtained for mammals whose blood contains carotene, however, in the only other investigation of the carotene levels in insect blood, Palmer & Knight (1924) found the astonishingly high value of 13,600 $\mu\text{g}/100\text{ ml}$ in the lymph of the potato beetle, *Leptinotarsa decemlineata* Say. (2) As the blood is bright green and carotene is the only pigment present it follows that it is highly likely that the carotene occurs as a protein complex.

Using the most rigid tests for detecting vitamin A, the antimony trichloride test and the correction procedure of Morton & Stubbs (1946), both carried out using a photoelectric spectrophotometer, it was never possible to demonstrate the presence of vitamin A in either species examined.

Brodskis (1944) and Brodskis & Rungs (1944) recently reported 'traces' of vitamin A in *Schistocerca*, it is suggested that, owing to the somewhat inadequate apparatus at their disposal, these investigators have been unable to distinguish between the colour produced by carotenoids with antimony trichloride and that produced by vitamin A.

The absence of vitamin A from locusts is not unexpected, for up to now the vitamin has never been unequivocally detected and is absent from *Taeniopoda auricornis* Walker (Giral, Giral & Giral, 1943), further, a number of insects can develop normally on diets from which vitamin A or carotene or both are completely absent (McCay, 1938, Bowers & McCay, 1940). The absence from locusts of vitamin A, coupled with the presence of astaxanthin, which is present in their eyes, strongly suggests that the pigment is the photoreceptor in locusts, for Wald (1943) has pointed out that astaxanthin is an almost universal photoreceptor in organisms which do not manufacture vitamin A.

SUMMARY

1 The carotenoids of the integument of the migratory locust (*Locusta migratoria migratorioides* R. & F.) and desert locust (*Schistocerca gregaria* Forsk.) are β carotene and astaxanthin, the latter occurs in the unesterified form.

2 Both pigments also occur in the eyes and the wings. Astaxanthin exists in the wings as a protein complex.

3 The presence of an astaxanthin protein complex has also been demonstrated in the wings of the red locust *Nomadacris septemfasciata* Serville.

4 Only β -carotene occurs in the fatty tissues, blood and eggs of both species. In the blood it is probably attached to a protein.

5 Preformed vitamin A does not occur in either *Locusta* or *Schistocerca*.

Thanks are due to the Anti Locust Research Centre for supplies of locusts and a grant towards expenses, to Drs B. P. Uvarov and D. L. Gunn of the Anti Locust Research Centre for advice and help willingly given, to Mr J. R. Bruce (Marine Biological Station, Port Erin, L.I.) for marine samples, and to Prof R. A. Morton for constant encouragement and advice.

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Some Observations on Astaxanthin Distribution in Marine Crustacea

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(Received 22 February 1949)

During an investigation into the carotenoids of two locust species (Goodwin & Srisukh, 1949) it became necessary to obtain an authentic specimen of astaxanthin for comparative purposes, such a sample was obtained from the lobster (*Homarus vulgaris* H M Edwards). As the isolation of this material proceeded it became apparent that the experience of Kuhn & Lederer (1933) on the distribution of free and esterified astaxanthin was not being reproduced, consequently the distribution was reinvestigated in some detail. A similar investigation was carried out on the prawn (*Nephrops norvegicus* L.) At the time the lobsters and prawns were being examined an opportunity arose to examine the pigment of the small red copepod, *Tigriopus fulvus* Fisch (Fraser, 1936).

EXPERIMENTAL

The first sample of astaxanthin used was obtained from old dry carapaces of lobster with which was mixed a small amount of carapaces of crab (*Cancer pagarus*). The carapaces were softened by placing in approx 0.2N-HCl for 24 hr to dissolve the CaCO_3 , filtered off, washed with water until free from acid, ground up in a mortar with acid washed silver sand and anhydrous Na_2SO_4 and extracted 3 times with acetone at a temperature below 30°. The combined acetone extracts were concentrated at the pump, diluted with 2 vol. of water and extracted with light petroleum. When a small sample of the light petroleum extract was shaken with 90% (v/v) aqueous methanol the pigment was almost completely hypophasic, this indicated that the pigment was unesterified, for esterified astaxanthin is epiphasic in this test (Kuhn, Lederer & Deutsch, 1933). A small yellow residue in the epiphase showed no selective absorption in the region 400–450 μ .

The light petroleum extract was then chromatographed using CaCO_3 (A.R.) as adsorbent. Development with light petroleum containing 4% (v/v) of ethanol resulted in the separation of three bands (1) orange red—most strongly adsorbed, (2) reddish pink, and (3) yellow. The yellow fraction, which was only small, was discarded, it was the

same pigment as that found in the epiphase in the partition test. Absorption spectrum measurements in four solvents indicated that the orange red pigment was astacin and the reddish pink pigment astaxanthin. Although the astaxanthin thus obtained was pure and was identical with the locust pigment, it was considered advisable to compare locust astaxanthin with astaxanthin prepared from fresh material. It was also important to examine fresh material because all our tests indicated that in the carapaces astaxanthin was present in the free form, although Kuhn & Lederer (1933) had claimed that the carapace pigment was esterified. In this experiment the presence of astacin in considerable amounts indicated that oxidative changes must have taken place in the stored carapaces and there was always the possibility that hydrolysis of the original ester (if ester were present) had also occurred.

Three fresh lobsters were killed by immersion in fresh water and the hypodermis removed carefully and as completely as possible from the carapace, the eggs of the two females and the hepatopancreases were also removed for examination.

The hypodermis on extraction with acetone in the manner just described yielded a pigment which was chromatographically homogeneous and epiphasic in a methanol light petroleum partition. It was the astaxanthin ester previously described by Kuhn and his co-workers, its spectral characteristics were recorded using a number of solvents.

The carapace was divided into two portions. One portion was extracted with acetone after it had first been softened by standing overnight in 2N HCl. The pigment extracted was not homogeneous, it consisted of two components, one epiphasic and the other, the major component, hypophasic. Neither component could be further fractionated by chromatography on weakened alumina (Goodwin & Srisukh, 1949). This experiment strongly indicated that the hypophasic fraction was obtained from the carapace itself and that the presence of a small amount of epiphasic pigment was due to contamination with small amounts of hypodermis. In order to confirm this, the second portion of carapace was allowed to stand for 48 hr in 0.02N HCl. This treatment softened the carapace and extracted a small proportion of the astaxanthin protein complex but not the free pigment. The resulting clear blue solution was filtered off and some

colourless interfering protein removed by addition of $(\text{NH}_4)_2\text{SO}_4$ to half saturation. The blue astaxanthin protein complex was then broken down by the addition of ethanol (equal vol), the solution immediately turned orange and the liberated pigment which remained dispersed in the solution was extracted with light petroleum. On shaking this petroleum solution with 90% (v/v) aqueous methanol the pigment was completely extracted by the methanol. This experiment clearly confirms that the carapace astaxanthin is unesterified.

Lobster eggs were ground up and allowed to stand for some days in contact with water. A green chromoprotein was extracted which exhibited an absorption spectrum with maxima at 476 and 660 $\text{m}\mu$, the spectrum was very similar in shape to that reported by Stern & Salomon (1938) who, however, recorded slightly different maxima, viz 470 and 640 $\text{m}\mu$. Denaturation of the chromoprotein by addition of acetone yielded unesterified astaxanthin, thus confirming the observations of Kuhn & Lederer (1933).

The hepatopancreases were extracted with acetone in the usual way and yielded only very small amounts of a pigment which was, as far as could be ascertained, β carotene.

Three fresh prawns (*Nephrops norvegicus* L.) were examined in the same way as lobsters. The results obtained were the same, i.e. the hypodermis contained esterified astaxanthin, whilst the carapace contained free astaxanthin attached to a protein.

The carotenoids from *Tigriopus fulvus* were extracted by acetone after grinding with sharp sand. The acetone was removed *in vacuo* and the residue dissolved in light petroleum (b.p. 40–60°), partitioned, and the two fractions purified by chromatography on weakened alumina. Using the methods described previously in this paper, these two pigments were identified as esterified and free astaxanthin, the latter is the major portion of the mixture.

Quantitative measurements were made in order to determine the amount of astaxanthin contained in male and female *T. fulvus*. Using samples of 100 animals, values obtained for males were 5.74 and 4.66 μg /animal and for females 6.0 and 5.77 μg . Gravid females are conspicuous by their egg sacs, consequently two lots of 100 gravid females were collected, separated from their eggs and the two portions examined separately. It was found that whilst females from which sacs had been removed contained 2.93 and 2.00 μg /animal, the sacs themselves contained astaxanthin to the extent of 2.89 and 1.58 μg /sac. From these limited experiments it appears that male and female *T. fulvus* contain about the same amount of pigment, but that gravid females lose about half their store to their eggs.

RESULTS AND DISCUSSION

Whilst Kuhn & Lederer's (1933) claim that the hypodermis of the lobster and prawn and ova of the lobster contain esterified and free astaxanthin, respectively, was confirmed, it was never possible to detect more than traces of astaxanthin ester in the carapaces of either species, the major pigment was always free astaxanthin. It can only be suggested that previous workers had investigated carapaces to which some hypodermis was still adhering. As the pigment concentration in the hypodermis is very much greater than in the carapace, the pigment

from small amounts of the former might well have masked that obtained from large amounts of the latter. It was found in the present investigation that complete removal of the hypodermis from the carapace was virtually impossible, and it is considered that the presence of small amounts of esterified pigment in our carapace extracts was due to traces of hypodermis. In order to eliminate the interference produced by the pigments of adhering hypodermis, air dried carapaces were ground with water containing traces of hydrochloric acid (about 0.02N) and allowed to stand. The astaxanthin protein complex was thus extracted without denaturation and the unconjugated hypodermal pigment was not. The resulting pale blue solution was denatured by the addition of ethanol or acetone, the colour changed to orange and the astaxanthin could be extracted with light petroleum. This pigment was completely hypophasic in the phase test, and no esterified astaxanthin could be detected.

Further, the absorption spectrum of the greenish astaxanthin protein complex (ovoverdin) which occurs in lobster eggs and which is soluble in water has been examined. The spectrum obtained agreed qualitatively with that previously recorded by Stern & Salomon (1938) in exhibiting a broad shallow band in the red region and a much sharper band in the blue green region of the spectrum. It is in the position of these bands that the present observations differ slightly from those of Stern & Salomon, they record λ_{max} at 640 and 470 $\text{m}\mu$, whilst we record them at 660 and 476 $\text{m}\mu$.

Light absorption data on astaxanthin are much less numerous than are those for astacin, Wald (1943) and Karrer & Wurgler (1943) record the spectrum in hexane, and Kuhn & Sørensen (1938) in pyridine. However, it is now apparent that the spectrum recorded by Kuhn & Sørensen is incorrect. In order to extend the data, the absorption maximum of astaxanthin in carbon disulphide, pyridine, glacial acetic acid and light petroleum (b.p. 40–60°) are recorded in Table 1. Similarly, only few data have

Table 1 Absorption maxima of free and esterified astaxanthin in various solvents

Solvent	Absorption maximum ($\text{m}\mu$)	
	Free astaxanthin	Esterified astaxanthin
Hexane	470	467–468
Light petroleum (b.p. 40–60°)	470	—
Pyridine	490–491	488
Carbon disulphide	505–506	503
Glacial acetic acid	483–485	481–482
Acetone	475	—

been produced on the spectra of astaxanthin esters (Wald, 1943, Kuhn & Sørensen, 1938) and these indicate that they are very close to that of the parent

compound. It has now been found that compared with free astaxanthin the esterified pigment occurring in lobster hypodermis shows an absorption band of exactly similar shape, but with its wavelength maximum shifted 1–2 μ to shorter wavelengths (Table 1). All the data were obtained using the Beckman photoelectric spectrophotometer.

SUMMARY

1 It has been confirmed that esterified astaxanthin occurs in the hypodermis of the lobster, *Homarus vulgaris* Edw and the prawn, *Nephrops norvegicus* L., and that the unesterified pigment occurs in the eggs of the lobster.

2 It has not been possible to confirm the presence of esterified astaxanthin in the carapaces of these species, from the evidence presented it is considered that the pigment is in fact free astaxanthin.

3 The lobster hepatopancreas contains only traces of β carotene.

4 Free and esterified astaxanthin, the former predominating, have been identified in the sea flea, *Tigriopus fulvus* Fisch. In gravid females about 50% of the pigment is in their eggs.

We are grateful to the Anti Locust Research Centre for a grant towards expenses, to Mr J R Bruce (Marine Biological Station, Port Erin, I o M) for specimens, and to Prof R A Morton for constant encouragement and advice.

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The Intermediary Metabolism of the Mammary Gland

2 RESPIRATION AND ACID PRODUCTION OF MAMMARY TISSUE DURING PREGNANCY, LACTATION AND INVOLUTION IN THE RAT

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(Received 11 March 1949)

In a previous paper (Folley & French, 1949c) we have shown that slices of lactating mammary tissue respire fairly actively in a medium containing glucose. In the rat the activity is less than that of nervous tissues and kidney, but is of the same order as that of liver, unlike that of liver, however, the respiration is markedly increased over endogenous values in the presence of glucose. The respiratory quotient (R Q) of lactating mammary tissue (in glucose) is well above unity in the mouse, rat, rabbit, and, to a lesser degree, the guinea pig, but below unity in ruminants (goat, cow).

Our results on the rat indicated that Q_{O_2} , and perhaps R Q, is lower in early than in full lactation. This preliminary finding was in line with that of Kleiber, Smith & Levy (1943), who reported a higher respiration for lactating tissue than for tissue taken from pregnant rats, provided the results were calculated to a dry tissue basis. On a moist tissue basis, however, there was no difference in metabolic rate at the end of pregnancy and at the twenty first day of lactation, because the dry matter content of

the gland was much higher in pregnancy than in lactation.

The relation between the functional activity of a tissue and its respiratory metabolism is of considerable interest, and the mammary gland readily lends itself to a study of this question. We have previously shown (Folley & French, 1949c) that, in the rat, experimental depression of lactation due to restriction of the food intake or to adrenalectomy, lowers Q_{O_2} , decreases the R Q to values near unity, and increases the aerobic glycolysis. Another approach to this question is to study the metabolism of mammary tissue at various stages of the lactational cycle, using the term in its widest sense to include late pregnancy and post lactational involution. The present paper reports results of such a study.

METHODS

Animals Hooded Norway rats undergoing their first lactations were used. The stock diet, fed *ad lib*, was as described previously (Cowie & Folley, 1948) save that 10 of the parts of whole wheat were replaced by wheat germ. All

litters were reduced to 8 (if possible 4 of each sex) at parturition. Groups were killed (by dislocation of the spine) on the twentieth day of pregnancy (i.e. 1-2 days before parturition), and on days 1, 8, 15 and 22 of lactation, litters being allowed access to the mothers up to the time of autopsy. Obviously the group killed on day 1 may have included some rats which would have failed to lactate successfully had they been allowed to survive. This could hardly have affected the mean results for this group of 10, however, since, of the rats set aside for this work, only about 10% failed to lactate. In order to allow the effects of weaning to be studied in comparison with those of continued suckling, a group was weaned on the twentieth day and killed 2 days later.

General. At autopsy the 3 abdominal* mammary glands from one side were carefully dissected off and weighed, after which samples were taken for determination of retained (extracellular) milk, by determination of the lactose content on a homogenate (Folley & Greenbaum, 1947), and of total dry matter content (Folley & Greenbaum, 1948). Tissue slices for the manometric experiments were cut from the other 3 glands as described previously (Folley & French, 1949c).

R.Q., Q_{O_2} (μ l O_2 /mg final dry wt/hr) and $Q_{O_2}^{std}$ were determined on duplicate slices from each rat by the method of Dickens & Simer (1931) using Dickens & Greville (1933) flasks. The gas phase was 5% CO_2 and 95% O_2 , and the medium the Ringer bicarbonate of Krebs & Henseleit (1932). In all experiments with substrate, 0.3% glucose was used. Determinations in duplicate without substrate were also performed on slices from about half the rats in each group.

RESULTS

Oxygen uptake. Group mean values for $-Q_{O_2}$ are given in Table 1. At the end of pregnancy $-Q_{O_2}$ (glucose) is low, but has increased considerably by

The increase in $-Q_{O_2}$ between the twentieth day of pregnancy and the first day of lactation is hardly affected by absence of substrate, but thereafter as lactation progresses $-Q_{O_2}$ increases but slightly, if at all, and it is doubtful whether there is a decrease at weaning. The values for the eighth, fifteenth and twenty second days of lactation confirm our previous finding (Folley & French, 1949c) that glucose markedly increases the respiration of mammary gland slices. Values for the ratio $\frac{Q_{O_2} \text{ (glucose)}}{Q_{O_2} \text{ (no substrate)}}$, given in Table 1, show that this is a property only of the fully lactating gland.

The interpretation of these results is complicated by the changes in the dry matter content of the mammary tissue (Table 2). Table 2 shows that the fully lactating gland contains less than half the dry matter of the gland at the end of pregnancy, the values agreeing very well with those of Kleiber *et al.* (1943). The question thus arises whether the increase in $-Q_{O_2}$ represents a real increase in respiration rate or is an artifact due to the disappearance of metabolically inert dry matter, such as fat or protein, contained in colostrum stored in the alveoli. On the other hand, there seems no doubt about the reality of the decrease in respiration following weaning, since the increase in the tissue dry matter at this time is relatively slight.

We have attempted to elucidate this problem by calculating for various stages the total respiration of the six abdominal glands, the weight of which, corrected for retained extracellular milk, should remain more or less constant over late pregnancy

Table 1. Respiratory metabolism of slices of rat mammary gland during pregnancy, lactation and involution

(Errors are indicated in this and succeeding tables by giving mean \pm S.E.M.)

Stage	Days	No of rats	Glucose (0.3%)		No substrate			Q_{O_2} (glucose) Q_{O_2} (no substrate)
			$-Q_{O_2}$	R.Q.	No of rats	$-Q_{O_2}$	R.Q.	
Pregnancy	20	10	1.3 ± 0.1	0.83 ± 0.01	5	1.5 ± 0.05	0.62 ± 0.03	0.87
Lactation	1	10	4.4 ± 0.3	1.00 ± 0.05	5	4.0 ± 0.3	0.73 ± 0.01	1.07
	8	8	7.1 ± 0.6	1.62 ± 0.03	4	4.5 ± 0.5	0.76 ± 0.01	1.58
	15	8	10.3 ± 0.4	1.60 ± 0.06	4	5.2 ± 0.4	0.78 ± 0.02	2.03
	22	8	9.6 ± 0.3	1.53 ± 0.03	4	6.3 ± 0.2	0.74 ± 0.02	1.49
Weaning	2	5	5.5 ± 0.9	0.76 ± 0.03	3	5.0	0.64	1.14

the first day of lactation. Thereafter the values rise steadily to a peak level, reached somewhere between the eighth and fifteenth days, which appears to be maintained sensibly constant until the twenty-second day, the slight drop at this time being not statistically significant. By contrast, glands from rats weaned on the twentieth day and killed on the twenty second day show a marked fall in respiration rate (see also Fig. 1).

* 'Abdominal' refers to the 2 abdominal and 4 inguinal glands.

and lactation, since glandular growth largely ceases by mid pregnancy (see Folley & Greenbaum, 1947). This approach seems preferable to an attempt simply to express the respiration rate on a moist tissue basis, since the nature of mammary gland slices (see Folley & French, 1949c) precludes accurate determination of their moist weights. The total respiration of the abdominal glands is easily calculated for 8, 15 and 22 days of lactation if Q_{O_2} , the total moist weight of the abdominal glands and their retained extracellular milk content, are known. The calculation involves

Table 2 *Total respiration of abdominal mammary glands of rats during pregnancy and lactation*

Stage	Days	No of rats	Mean body wt (g)	Moist wt of 3 abdominal mammary glands (including milk) (g)	Milk content of mammary tissue* (%)	Total dry matter content of mammary tissue (including milk) (%)	Dry matter content of 'milk free' mammary tissue (calc)† (%)	Total respiration of 6 abdominal mammae‡ (μl O ₂ /hr)
Pregnancy	20	10	290	2.56	12.8±0.7	58.1±1.7	—	3,870§
Lactation	1	10	224	3.35	40.4±3.1	37.2±1.8	—	—
	8	8	248	3.12	37.8±3.0	28.6±0.6	30.4	8,370
	15	8	250	3.75	42.2±2.5	25.7±0.3	25.8	11,500
	22	8	250	3.49	57.6±2.1	20.0±0.3	26.6	7,540
Weaning	2	5	251	5.58	41.5±3.6	31.2±0.6	—	—

* Lactose content of rat milk taken as 3.23% (Folley & Greenbaum, 1947)

† Dry matter content of rat milk taken as 25.60% (Folley & Greenbaum, 1948)

‡ Calculated on the assumption that all the milk in a slice is leached out during the manometric determination

§ Calculated on the assumption that no colostrum is leached out from a slice during the manometric determination.

a number of reasonable assumptions, the most important of which are the following (a) that the method of Folley & Greenbaum (1947) for calculating the extracellular milk content of mammary tissue from the lactose content of tissue homogenates is reasonably valid, (b) that the variations in the total solids and lactose contents of the milk over the period of lactation in question are negligible, and (c) that all extracellular milk is leached out from the slice during the respiration experiment. In calculating the milk content of the tissue at these stages the lactose content of rat milk was taken as 3.23% (Folley & Greenbaum, 1947) and the total solids content as 25.60% (Folley & Greenbaum, 1948).

These assumptions are, however, not valid for the gland in late pregnancy because (a) the gland then contains colostrum, the composition of which is unknown for the rat, but which by analogy with what is known for the heifer (Engel & Schlag, 1925) is almost certain to contain a very large amount of solids and little lactose, and (b) it seems probable that much of the colostrum will remain in the slice throughout the manometric determination. The total respiration of the abdominal glands for the twentieth day of pregnancy was therefore calculated on the assumption that no colostrum is leached out. This has the advantage of giving a maximum value for the total respiration since if, as is probable, some colostrum is lost during the measurements, the figure given is an overestimate. No such calculation was attempted for the first day of lactation since this is probably a period of transition from colostrum to milk, involving unknown and rapidly changing conditions to which no set of assumptions seemed to apply.

The values for the total respiration given in Table 2 indicate an increase of the order of threefold between the end of pregnancy and the fifteenth day of lactation, the increase being somewhat less at the eighth and twenty second days.

Respiratory quotient Group mean values are given in Table 1, they are of course independent of changes

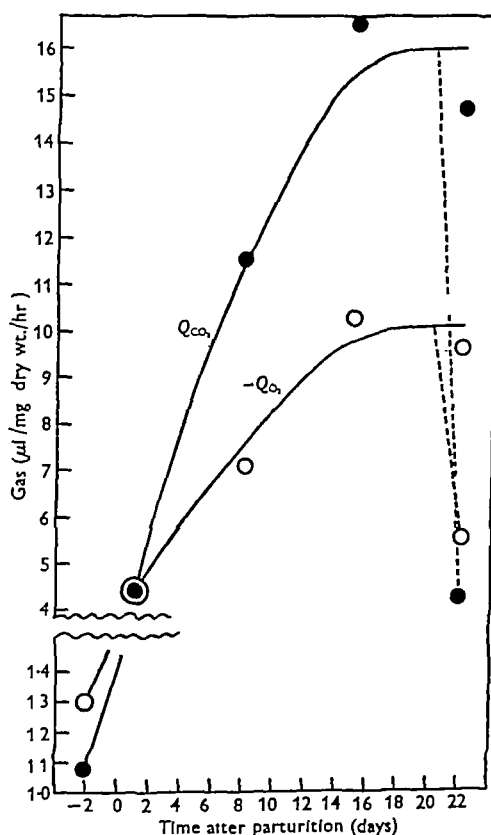


Fig 1 Gas exchange of rat mammary gland slices during late pregnancy, lactation and after weaning. The points at the ends of the dotted lines refer to groups of rats weaned at the twentieth day of lactation.

in the dry matter or milk content of the tissue. With glucose as substrate the RQ is well below unity in late pregnancy, but has risen to unity on the first day

of lactation. By the eighth day the R/Q has increased to 1.6, a value which is more or less maintained throughout lactation. This value is slightly higher than our previous value for rat mammary gland in full lactation (Folley & French, 1949c). After weaning for 2 days the R/Q has dropped below unity to a value similar to that in late pregnancy. The striking increase in CO_2 output, the increase in Q_{CO_2} above $-Q_{O_2}$ near parturition and the reversal of this change following weaning, which these findings imply, are illustrated in Fig. 1. In the absence of substrate the R/Q is below unity throughout, although the values for fully lactating tissue are a little greater than those for tissue taken in late pregnancy or after weaning.

Aerobic acid production. Changes in aerobic acid production (Table 3) are mainly considered in terms of ratios of quotients so as to eliminate complications due to changes in the dry matter and milk content of tissue. We have previously pointed out (Folley & French, 1949c) that the acid produced by mammary gland slices probably includes acids other than lactic acid.

in autoglycolysis relative to the glycolysis as the secretory activity of the tissue diminishes.

DISCUSSION

The present results show that, allowing for simultaneous changes in the dry-matter content of the tissue, the onset of lactation in the rat and the subsequent progressive increase in milk yield (see data of Brody & Nisbet, 1938) are correlated with an increase in the metabolic rate of mammary tissue. Our values for Q_{O_2} are at all stages two to three times as great as those of Kleiber *et al.* (1943), possibly because those workers did not use thin slices, so that their tissue may not have been in equilibrium with oxygen. Studies of the oxygen uptake of the ruminant udder by the arteriovenous method show on the whole (e.g. the results of Reineke, Stonecipher & Turner, 1941, on the goat) very little difference between dry and lactating udders as regards arteriovenous differences in oxygen. Our findings on the rat indicate that the enhanced energy consumption in lactation is met by an increase in respiration rather than glycolysis, so in the ruminant, and probably

Table 3. *Aerobic acid production of rat mammary gland during pregnancy, lactation and involution*

Stage	Days	No. of rats	$Q_{O_2}^a$ (glucose)	$-Q_{O_2}$ (glucose)	No. of rats	$-Q_{O_2}$ (no substrate)	$Q_{O_2}^a$ (glucose)
				$Q_{O_2}^a$ (glucose)		$Q_{O_2}^a$ (no substrate)	$Q_{O_2}^a$ (no substrate)
Pregnancy	20	10	0.9 ± 0.1	1.45	5	4.47	2.77
Lactation	1	10	1.4 ± 0.1	3.57	5	9.04	2.90
	8	8	1.8 ± 0.2	4.09	4	9.28	3.76
	15	8	2.4 ± 0.5	5.25	4	12.75	3.08
	22	8	2.0 ± 0.2	5.16	4	6.37	2.40
Weaning	2	5	3.5 ± 0.3	1.55	3	3.51	2.63

The ratio $-Q_{O_2}/Q_{O_2}^a$ (glucose), which gives a measure of the relative changes in respiration and aerobic glycolysis, increases from about 1.5 at the end of pregnancy to a maximum value greater than 5 at the fifteenth day of lactation. Since the total respiration of the abdominal glands increases at least threefold over this period (Table 2), it would seem that the increasing activity of the mammary gland is not accompanied by any appreciable increase in aerobic glycolysis. The extra energy requirements of lactation appear to be met solely by increased oxidation. Table 3 shows that $Q_{O_2}^a$ increases after weaning, since there is also an appreciable increase in the dry matter content of the tissue at this time (Table 2), the actual increase in glycolysis at weaning is probably somewhat greater than indicated by the quotients.

Similar results were obtained in the absence of substrate, although under these conditions the ratio of respiration to glycolysis is higher. The values for the ratio $\frac{Q_{O_2}^a \text{ (glucose)}}{Q_{O_2}^a \text{ (no substrate)}}$ show relatively little change, but there is an indication of a slight increase

in other mammals as well, the increased energy requirements of lactation may involve an increase in blood flow. Jung (1932) reported that the blood flow through the lactating goat udder was about four times the value for the dry gland, an increase in fair agreement with our estimate of a threefold increase in the total respiration of the abdominal glands in the rat.

Even more significant, because in a sense they are qualitative rather than purely quantitative, are the changes in R/Q (in glucose). The R/Q , well below unity at the end of pregnancy, has risen to unity shortly after parturition and thereafter rises to a value of 1.5–1.6 which is maintained throughout lactation. Despite criticisms of the validity of the R/Q as an indicator of metabolic processes (Soskin, 1941), it seems safe to conclude that the change in R/Q of mammary tissue at parturition implies a profound change in the intermediary metabolism of the mammary gland at the start of secretion.

A brief consideration of what this change implies may not be out of place. In previous papers (Folley & French, 1948a, 1949c) we concluded that the high

in vitro R Q of lactating mammary tissue indicates the synthesis of fat from oxygen-rich materials. It is probable, however, that even in late pregnancy, when the R Q is low, some fat synthesis is proceeding, and indeed Popjak & Beeckmans (1949) has demonstrated the incorporation of ^{14}C into the fatty acids of neutral fat isolated from the mammae of pregnant rabbits given labelled acetate. There thus appears to be a turnover of fat in the gland even during pregnancy, but the high R Q following parturition given by slices of ruminant udder in the presence of acetate and of non ruminant gland in the presence of glucose (Folley & French, 1948*b*, 1949*a*) undoubtedly indicates net synthesis of fat by lactating tissue. We have suggested that part at least of the milk fat, perhaps particularly the shorter chain fatty acids which distinguish it from body fat, is synthesized in the mammary gland itself from acetate (Folley & French, 1948*b*, 1949*a*) which the ruminant absorbs in large quantities from the rumen. Since Bloch & Rittenberg (1945) have shown in the rat that considerable amounts of acetate are continually produced, it seems likely that acetate is a substrate for synthesis of milk fat by the mammary gland in all mammals. The above mentioned experiments of Popjak & Beeckmans (1949) confirm this for the rabbit. The apparent ability, as indicated by the high R Q, of mammary slices from non ruminants, to effect net synthesis of fat from carbohydrate *in vitro* (Folley & French, 1948*a*, 1949*c*) would be explicable if the pathway from carbohydrate to fat passes through acetate. Bloch (1947) does not favour this possibility, but the position is admittedly rather obscure, and further *in vitro* work on the mammary gland might well help in clarification. The main difficulty hitherto has been the inability of lactating tissue from non ruminants to utilize acetate *in vitro* in contrast to slices from ruminant udders (Folley & French, 1948*b*, 1949*a*), but in recent experiments (Folley & French, 1949*b*) we have shown that lactating mammary slices from rabbit and rat will utilize acetate in presence of small concentrations of glucose, the R Q being above unity, and in the rabbit often greater than in the presence of glucose alone. It seems that mammary slices from various species are subject to different limiting conditions, and it may be that non ruminant mammary tissue, which contains very little glycogen (Folley & French, unpublished), requires glucose to provide the glycerol necessary for glyceride synthesis, a process which may well favour the synthesis of fatty acids. Other possibilities are that glucose is necessary to provide additional carbon for fatty acid synthesis, as suggested by Bloch (1947), or to provide energy for the activation of acetate.

The onset of lactation involves a change from a condition characteristic of late pregnancy in which, although cytologically the secretory phase has begun,

the product bears little resemblance in composition to milk, to the post parturient state in which, provided suckling proceeds, large quantities of normal milk are secreted. These events are accompanied by the following changes in the *in vitro* metabolic properties of the rat mammary gland: (a) the metabolic rate increases to an extent difficult to assess from our data, but probably at least threefold, (b) the slices acquire the property of responding to glucose in the medium by an increase in respiration, (c) the R Q increases above unity, and (d) the ratio of respiration to glycolysis increases.

The initiation of lactation is under endocrine control, the chief feature being an increased release of prolactin, and perhaps of other hormones concerned in lactation, by the anterior pituitary (see review by Folley, 1947*a*), and it would therefore seem likely that a close relationship must exist between the lactogenic hormone or hormone complex and the changes in the mammary gland metabolism occurring at parturition.

The changes which follow weaning are also of interest in this connexion. Within a short time of removing the litter the mammae exhibit changes in metabolism in the opposite direction from those which occur at parturition, the respiration decreases and, more significant, the R Q quickly drops below unity. Weaning also causes a definite increase in the apparent aerobic glycolysis similar to that shown previously (Folley & French, 1949*c*) to be associated with the partial inhibition of lactation resulting from inanition or adrenalectomy. Changes in mammary metabolism following weaning may be due to (a) the loss of the suckling stimulus, and (b) the effects, chemical or physical, of non removal of milk. No attempt has been made in the present work to assess the relative role of these two primary factors, but obvious test experiments, utilizing the techniques of Selye (1934), suggest themselves. The suckling stimulus is believed to influence the function of the mammary gland through a neurohormonal arc involving as its final, centrifugal link the release of prolactin by the anterior hypophysis (see Folley, 1947*b*, for review), and the experiments of Selye (1934), and others, suggest that removal of the suckling stimulus may be the more important of the two above mentioned factors, at any rate in the early stages of weaning. Thus the changes in the R Q of mammary tissue at parturition and after weaning alike raise the question how far the physiological action of prolactin on the mammary epithelium, an action which the experiments of Lyons (1942) and Meites & Turner (1948) involving intramammary duct injection of prolactin show to be direct, is bound up with the promotion of reactions leading to synthesis of fat from oxygen rich materials. Mammary tissue may well prove particularly useful for an attack on this aspect of what is undoubtedly the

outstanding problem of endocrinology, the hormone enzyme relationship

The results for the unweaned group at 22 days of lactation seem to indicate that the respiration and R Q have begun to decline, but in order to study the normal course of lactation beyond this point it would be necessary to provide fresh litters so as to eliminate the effect of self weaning of the young (Incidentally, self weaning is shown by the increased milk content of the glands at 22 days, Table 2)

In conclusion, we may refer to the value of a more or less complete picture of changes in the respiratory metabolism of the mammary gland in relation to various phases of its physiological activity, such as is provided by this work, as forming a background for further studies of the biochemical mechanisms involved in the synthesis of milk

SUMMARY

1 The respiratory metabolism and acid production of slices of rat mammary gland in the presence and absence of glucose have been studied during pregnancy, lactation and following weaning

2 $-Q_{O_2}$ is low at the end of pregnancy, but has increased considerably on the day following parturition, thereafter the values rise to a maximum value of approximately 10 at mid lactation Interpretation of these changes is complicated by changes in the dry matter content of the tissue, which by mid lactation has fallen to less than half of the value at the end of pregnancy However, calculation of the total respiration of the abdominal mammary glands

indicates that the initiation and subsequent increase in the intensity of lactation are accompanied by a true increase in the respiration

3 The effect of glucose in increasing the respiration of mammary gland slices is only seen in lactating tissue

4 The R Q (in glucose) is well below unity at the end of pregnancy, but has risen to unity on the day following parturition, thereafter it rises to a maximum of approximately 1.6, which is maintained throughout most of the lactation period In absence of substrate, the R Q remains below unity throughout lactation

5 Lactating gland shows a higher value for $Q_{O_2}^0$ than the gland at the end of pregnancy, the increase is, however, largely an artifact due to the changes in the dry matter content of the tissue The extra energy requirements of the lactating gland seem to be met solely by increase in respiration

6 Weaning is followed by a sharp fall in respiration, the change in Q_{O_2} being too great to be attributed to the relatively slight increase in the dry matter content of the tissue which occurs at this time Further, the R Q decreases below unity and the apparent aerobic glycolysis rises

7 The significance of the results is discussed in the light of possible mechanisms of fat synthesis in the mammary gland, and of relationships between anterior pituitary hormones and changes in mammary gland metabolism

We are indebted to Dr S. K. Kon for extending to us the facilities of his rat colony, to Dr A. L. Greenbaum for some lactose determinations, and to Mr S. C. Watson for skilled technical assistance

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Tryptophan and the Biosynthesis of Nicotinamide*

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(Received 17 January 1949)

There has been much discussion as to how tryptophan affects nicotinamide metabolism. It is generally believed that a direct conversion of tryptophan into nicotinamide takes place and many experiments have been carried out to elucidate the route of this conversion. Ellinger & Abdel Kader (1949c) observed that in *Escherichia coli* the biosynthesis of nicotinamide from ammonium lactate and ornithine was completely inhibited by DL 2, 4, 5- and 7-methyltryptophans in 2 mM concentrations, growth not being affected. This inhibition suggested, in the light of Fildes's (1940) theory of metabolite antagonism, that tryptophan is actively involved in the biosynthesis of nicotinamide. Heidelberger, Gullberg, Morgan & Lepkowsky (1948) found recently that after administration of DL tryptophan labelled in the β -position of the side chain with ^{14}C to dogs, rabbits and rats, kynurenine and kynurenic acid eliminated in the urine contained the labelled carbon atom, but that the urinary nicotinamide methochloride was free from radioactivity. These results suggested the possibility of tryptophan not being directly converted into nicotinamide, but having a catalytic coenzyme like action in the biosynthesis of nicotinamide. The present paper deals with experiments to test this hypothesis.

EXPERIMENTAL

Experiments were carried out *in vitro* with mixed cultures from rat caecum content and with pure cultures of *E. coli*, and *in vivo* with rats.

Material used. The mixed cultures of rat caecum content were prepared in the following way: rats which had been found to have a high nicotinamide methochloride elimination were gassed, and immediately afterwards the content of the caecum was placed in weighing bottles under sterile conditions. One portion (about 200 mg wet weight) was ground in a mortar with 10 ml peptone water to form a homogeneous suspension and the rest was dried to constant weight in order to calculate the dry weight of the suspended material. From the suspension and from dilutions prepared from it agar plates were inoculated and smears were made in order to obtain qualitative and quantitative information about the organism present. There was a great variety of organisms contained in the mixed cultures. The most frequent types

were hitherto unidentified cocci growing on agar in minute colonies, various strains of streptococci, staphylococci and coliform bacilli. Their relative frequency in the caecum content was about 8000:160:80:1, respectively. In addition, a number of other organisms (acidophili, *Proteus*, Gram positive cocci and diplococci, Gram negative oval cocci and Gram positive bacilli) were occasionally found. The *E. coli* used were the strains 3c and 4c isolated from human and rat faeces respectively, which were classified as type I faecal (Ministry of Health, 1939). The bacteria were grown on agar slopes and fresh cultures were made in ammonium lactate for inoculation.

Six rats used in the *in vivo* experiments were cross bred from the hooded Lister stock and P. Ellinger's albino stock, of both sexes, about 4 months old and weighing 280–320 g. They were kept on the mixed diet described previously (Ellinger, Fraenkel & Abdel Kader, 1947). Their average daily nicotinamide methochloride output and their response to nicotinamide had been examined for several months.

Salt solutions. The following solutions were used for incubation: (1) saline solution 0.89% (w/v) NaCl, (2) ammonium lactate solution (Fildes, 1938), (3) saline phosphate solution KH_2PO_4 , 4.5 g, NaCl, 1.0 g, MgSO_4 , 0.02 g, FeSO_4 , 0.02 g, in 400 ml. water, adjusted with NaOH to pH 7.6, 4 ml. of this solution were made up to 10 ml. with water, (4) saline phosphate glucose solution: glucose was added to solution (3) to a final concentration of 1 mM., (5) casein hydrolysate solution (Barton Wright, 1944).

Analytical methods. Estimations were carried out in duplicate. The growth of bacteria was measured by comparing the opacity of a suspension with that of a standard (Brown, 1919). Nicotinamide was assayed microbiologically (Barton Wright, 1944) either in the medium alone after removing the cells on the centrifuge, or in the medium and organisms after autoclaving. Nicotinamide methochloride was measured by the acetone method (Huff & Perlzweig, 1947). Free tryptophan (without indole) was estimated by the method of Horn & Jones (1945). DL-Tryptophan from different sources was used for the rat experiments, commercial samples from Glaxo Laboratories Ltd. and from Roche Products Ltd., unpurified, for bacterial experiments the same samples, unpurified and purified by repeated recrystallization, and one sample prepared and kindly supplied by Dr R. L. M. Syngé. No difference was observed in their action.

General arrangement of the experiments

In order to measure the consumption of tryptophan during the synthesis of nicotinamide, casein hydrolysate and ammonium lactate solutions without or with the addition of 2 mM DL-tryptophan, and 2 mM DL-ornithine dihydrochloride were inoculated with a heavy inoculum of pure cultures of *E. coli* 4c, the saline washings of broken *E. coli* 4c or a mixed culture from the rat caecum and incubated at

* Some of the results presented in this paper were communicated to the Biochemical Society on 4 December 1948 (Ellinger & Abdel Kader, 1948).

37° for 48 hr Nicotinamide and free tryptophan in the solution were measured before and after incubation

The effect of washing the cells on their ability to synthesize nicotinamide from ammonium lactate and on the effect of tryptophan on this synthesis was studied. A 48 hr culture of *E. coli* in casein hydrolysate was centrifuged and the organisms were washed with water. Samples of the suspension (75×10^6 organisms/ml) were examined for nicotinamide production in an ammonium lactate medium in the absence and presence of 2 mM DL-tryptophan, of the unwashed cells and 3, 6 and 9 times washed cells. The samples were prepared by adding 0.5 ml of a culture containing 15×10^6 organisms/ml to 9.5 ml of salt solution.

The dependence of the nicotinamide formation by washed *E. coli* on the concentration of the tryptophan was examined in the following way. *E. coli* 4c, washed 3 times with water, were suspended in ammonium lactate solution containing various concentrations of DL-tryptophan, incubated at 37° for 48 hr and the nicotinamide content was measured. In order to obtain more detailed information on the biosynthesis of nicotinamide when the enzyme system involved was freed from all possible substrates, fresh *E. coli* cells washed 4 times with water and then suspended in saline were disintegrated mechanically (Curran & Evans, 1942). A portion of the broken cells was washed several times with saline, part of the washings was dialysed through cellophane against saline under sterile conditions at room temperature for 48 hr. The outside solution was changed 3 times and continuously stirred mechanically during the dialysis. The completeness of the disintegration was examined by plating and taking viable counts at the various steps. The washed cells, the broken cells, the saline washings before and after dialysis and the exhaustively washed cell fragments were used for one or other of the following experiments. They were incubated in either saline phosphate, saline glucose phosphate or ammonium lactate solutions in the absence or presence of DL-tryptophan, DL-ornithine dihydrochloride or both at 37° for 48 hr. The nicotinamide content of the solution was estimated after incubation.

Rat experiments. The rats were kept in metabolism cages, the urine was collected daily and nicotinamide methochloride elimination was determined. At intervals of 3 or 4 days the rats were given either DL-tryptophan or nicotinamide in doses rising from 20 to 400 mg and from 2.5 to 50 mg respectively. These compounds were administered either orally by mixing them thoroughly with the food and con-

trolling the completeness of the consumption, or intraperitoneally.

RESULTS

Experiments with bacteria

In all microbiological experiments two to three parallel experiments were carried out which gave similar results to those reported in detail. Experiments to relate the consumption of tryptophan to the synthesis of nicotinamide during incubation are given in Table 1. Although the growth of the pure cultures of *E. coli* used was good, no marked diminution of tryptophan was observed with the method used, which is rather insensitive but is probably the best existing procedure. 20 µg/ml is the lower limit of assay and the standard deviation is about $\pm 5\%$. By concentrating the salt solutions to be examined *in vacuo*, amounts of 1 µg/ml might be measured with a standard deviation of about $\pm 10\%$. Similar experiments carried out with washed cells and washings of broken cells also showed no detectable consumption of tryptophan during incubation, while measurable amounts of nicotinamide were formed. However, the amounts of nicotinamide formed are very small compared with those of the tryptophan present in the medium and the method used for the former is about ten thousand times more sensitive than that employed for tryptophan. The results with pure *E. coli* are therefore not conclusive. By contrast the tryptophan consumption of mixed cultures (Table 1) during nicotinamide synthesis in both ammonium lactate ornithine tryptophan and in casein hydrolysate solution is definite and outside the limit of error of the method. Growth was also considerable in these experiments.

When *E. coli* cells were washed repeatedly with water they gradually produced less nicotinamide (Table 2). The nicotinamide synthesis, however, was always restored to the level of the unwashed cells when 2 mM tryptophan was added, with the washed cells no growth was observed. The relative effect of tryptophan on the synthesis of nicotinamide as

Table 1 Content of tryptophan and nicotinamide of salt solutions before and after incubation

Inoculum	Salt solution	Tryptophan content of salt solution (µg/ml)		Nicotinamide content of salt solution and organisms (µg/ml)	
		Before incubation	After incubation	Before incubation	After incubation
<i>E. coli</i> 4c	Ammonium lactate	<20	<20	10	32
	Ammonium lactate + ornithine (2 mM) + DL-tryptophan (2.2 mM)	442.0	441.4	8	49
	Casein hydrolysate	87.6	87.7	25	40
	Ammonium lactate + DL-tryptophan (1.9 mM)	382.8	383.1	0	27
Saline washings of broken up <i>E. coli</i> 4c	Ammonium lactate	<20	<20	15	31
Mixed cultures from rat caecum	Ammonium lactate + DL-ornithine (2 mM) + DL-tryptophan (2.2 mM)	440.0	385.4	12	33
	Casein hydrolysate	87.7	71.2	10	25
					18

compared with the control (without tryptophan) is greater in washed than in untreated cells. This effect is presumably due to the decreased nicotinamide synthesizing activity of the cells, caused by the washing.

Concentrations of DL tryptophan up to about 0.1 mM had no stimulating effect on the nicotinamide synthesis by washed *E. coli* in ammonium lactate solution. At a concentration of 0.3 mM a marked increase was observed and the stimulating effect reached its maximum at concentrations of 1–2 mM. Higher concentrations did not produce any further increase in nicotinamide synthesis.

Table 2 *The effect of repeated washings of E. coli 4c on the nicotinamide synthesis in ammonium lactate solution and on its increase by tryptophan*

(In all tubes the concentration of the cells was about 40×10^8 organisms/ml.)

No. of washings Salt solution	Nicotinamide content of salt solutions and organisms ($\mu\text{g/ml}$)			
	0	3	6	9
Ammonium lactate	28	15	7.5	5.0
Ammonium lactate + 2 mM DL-tryptophan	28	28	29	28

Table 3 *The effect of various concentrations of DL-tryptophan on the nicotinamide synthesis in ammonium lactate solution by washed E. coli 4c*

Tryptophan concentration (mM)	Nicotinamide content of salt solution and organisms ($\mu\text{g/ml}$)
0	10
0.1	11
0.3	15
1.0	25
2.0	25
5.0	24
10.0	24

Table 4 *Nicotinamide synthesis by washed and by broken E. coli 4c in ammonium lactate solution in the absence or presence of ornithine, tryptophan or both*

Compound added	Washed <i>E. coli</i> , nicotinamide content of salt solution and organisms ($\mu\text{g/ml}$)	Broken <i>E. coli</i> , nicotinamide content of salt solution ($\mu\text{g/ml}$)
None	6.3	7.5
DL-Ornithine (2 mM)	50.0	50.0
DL-Tryptophan (1 mM)	32.5	32.5
DL-Tryptophan (2 mM)	38.5	35.5
DL-Ornithine (2 mM) + DL- Tryptophan (1 mM)	49.0	48.0
DL-Ornithine (2 mM) + DL- Tryptophan (2 mM)	49.0	50.6

Synthesis of nicotinamide was also observed on incubation of washed *E. coli*, broken cells (Table 4),

and undialysed or dialysed washings (Table 5) of disintegrated cells, in ammonium lactate solution alone or with either tryptophan, ornithine or both tryptophan and ornithine. Ornithine increased the synthesis considerably as did tryptophan, but the synthesis in the presence of ornithine was not markedly affected by simultaneous addition of tryptophan. When the same experiment was carried out in saline phosphate solution no nicotinamide was formed in the absence or presence of tryptophan alone, but nicotinamide was produced in the presence of ornithine by undialysed and dialysed cell washings. No nicotinamide was formed in saline phosphate solution containing tryptophan by washed cells even in the presence of glucose, or by saline washings of broken cells. No nicotinamide was formed by washed cells, broken cells or cell washings in 1% saline solution in the presence of ornithine or tryptophan or both of them. No nicotinamide was formed by the exhaustively washed cell fragments in either solution with or without added tryptophan or ornithine.

The enzyme systems involved in the nicotinamide synthesis were contained in the saline washings of the broken cells and retained their nicotinamide synthesizing activity after dialysis. *E. coli* 3c behaved similarly to *E. coli* 4c.

Experiments with rats

When rats (Table 6) were given rising doses of nicotinamide by mouth the urinary elimination of nicotinamide methochloride increased proportionately to the dose administered. The increase varied individually in the different rats, but in the same rat it rose in proportion to the dose given for doses between 2.5 and 50 mg. The response to intraperitoneally administered nicotinamide was in all six rats lower than when nicotinamide was given by mouth. When tryptophan was given by mouth in doses of 50 mg and more, there was an increase in nicotinamide methochloride output proportional to the dose administered up to about 200 mg. Above this level no further increase of nicotinamide methochloride elimination occurred in any of the six rats. When tryptophan was injected intraperitoneally no increased nicotinamide methochloride output was observed in either rat after doses up to 100 mg and only a very slight increase in two rats after doses of 200 mg.

DISCUSSION

Ellinger & Abdel Kader (1949a) showed that tryptophan increased the nicotinamide synthesis by mixed cultures from the rat faeces or caecum content, but not that by pure cultures of *E. coli*. This action had been attributed to an ability of some organisms of the mixed cultures, but missing from *E. coli*, to split the tryptophan molecule in such a way that the

Table 5 Nicotinamide synthesis by undialysed saline washings of broken cells of *E. coli* 3c and 4c and dialysed washings of *E. coli* 4c in ammonium lactate and saline phosphate solutions in the absence and presence of DL ornithine and DL tryptophan

Salt solutions	Compound added	Nicotinamide content of washings		
		Undialysed <i>E. coli</i> 3c (μg /ml)	Undialysed <i>B. coli</i> 4c (μg /ml.)	Dialysed <i>E. coli</i> 4c (μg /ml)
Ammonium lactate	None	6.3	10.5	12.5
	DL-Ornithine (2 mM)	35.0	40.0	33.5
	DL-Tryptophan (2 mM)	25.0	37.0	40.0
	DL-Ornithine (2 mM) + DL-tryptophan (2 mM)	35.0	40.0	35.0
Saline phosphate	None	0	0	0
	DL-Ornithine (2 mM)	15.0	25.0	12.5
	DL-Tryptophan (2 mM)	0	0	0
	DL-Ornithine (2 mM) + DL-tryptophan (2 mM)	15.0	26.0	12.5

Table 6 The urinary elimination by rats of nicotinamide methochloride following oral or parenteral administration of nicotinamide or DL tryptophan

Compound tested	Dose (mg)	Route of administration	Increase in nicotinamide methochloride output (mg)			
			Rat 1	Rat 2	Rat 3	Rat 4
Nicotinamide	2.5	Oral	1.704	1.099	1.068	2.062
	5.0	Oral	5.128	2.423	2.524	5.146
	10.0	Oral	8.138	5.871	6.249	9.402
	20.0	Oral	15.133	10.210	11.947	16.670
	50.0	Oral	28.389	18.280	28.885	40.117
	10.0	Intraperitoneal	2.931	4.136	5.424	5.688
DL-Tryptophan	20	Oral	0	0	0	0
	50	Oral	1.246	0.631	0.300	1.530
	100	Oral	3.132	1.395	0.450	2.400
	200	Oral	5.909	2.565	1.149	4.076
	400	Oral	6.493	2.550	1.125	3.039
	50	Intraperitoneal	0	0	0	0
	100	Intraperitoneal	0	0	0	0
	200	Intraperitoneal	0.840	0.085	0	0

Experiments with two more rats revealed essentially similar results

latter could be utilized by *E. coli* for the synthesis of nicotinamide. When washed coli, instead of untreated coli, were examined for their ability to synthesize nicotinamide and the action of tryptophan on this synthesis, it was found that tryptophan increased nicotinamide production by the washed cells as compared with the control in ammonium lactate solution in a similar way as was found with mixed cultures. The systematic examination of the effect of repeated washings on the nicotinamide production by *E. coli* and on the tryptophan action on the synthesis (Table 2) showed that washing gradually diminished the synthesizing power of the organism and that this was restored by added tryptophan. The apparent relative increase in nicotinamide synthesis by added tryptophan as compared with that in ammonium lactate alone was, therefore, due to the restoration of tryptophan removed by washing. This fact indicated that the stimulating effect of tryptophan on the nicotinamide synthesis by mixed cultures might possibly be due to a reduced tryptophan concentration of the medium. That this

wasso, was shown by investigation of the tryptophan content after incubation of mixed cultures (Table 1). This was considerably reduced during incubation and nicotinamide synthesis. Whether the unchanged tryptophan content of the salt solutions of pure *E. coli* cultures during growth and nicotinamide synthesis was due to tryptophan formation equal to tryptophan destruction or to the fact that changes were too small to be detected by the method of assay could not be decided.

The experiments show, at least, that with pure cultures of *E. coli* nicotinamide synthesis is not accompanied by a marked consumption of tryptophan as in the case of mixed cultures. The concentration of tryptophan optimal for maximum nicotinamide formation was found to be 1–2 mM (Table 3) and the concentration of about 2 mM was, therefore, used in all experiments.

The facts discussed so far, namely, that tryptophan stimulates nicotinamide synthesis by *E. coli* in ammonium lactate solution only when its concentration in the cells is suboptimal, that its concentration

in mixed cultures is suboptimal and consequently the nicotinamide synthesis by these mixed cultures is stimulated by added tryptophan and that rising concentrations of added tryptophan increase nicotinamide synthesis by washed coli cells proportionally to its concentration only until the optimal level is reached, can best be explained by a catalytic action of tryptophan in the nicotinamide synthesis by *E. coli*. Moreover, the fact that no nicotinamide is formed by undialysed or dialysed washings of broken cells when tryptophan, but no other source of nitrogen, is present in the salt solution (Table 5) supports this hypothesis.

As had been shown (Ellinger & Abdel Kader, 1949a) nicotinamide synthesis by *E. coli* is independent of the intactness of the cell structure. This is confirmed by the results shown in Table 5. Moreover, the fact that the enzyme system is contained in the washings of the broken cells and can be dialysed without loss of activity, made it possible to remove all soluble and dialysable substrates from the enzymes.

It had been suggested that ornithine might be an intermediate in the biosynthesis of nicotinamide (Klein & Linser, 1932; Guggenheim, 1940; Ellinger & Abdel Kader, 1949a). In the presence of ornithine, tryptophan had very little or no effect on nicotinamide synthesis by either of the coli preparations. If ornithine is formed as an intermediate the findings would mean that tryptophan is mainly involved in the early stages of the nicotinamide synthesis, i.e. from ammonia and lactate to ornithine.

The experiments on rats (Table 6) confirmed the findings of Schweigert & Pearson (1947) and of Ellinger & Abdel Kader (1949a) that tryptophan increases the nicotinamide methochloride output to a far higher degree when given orally than parenterally. The differences were, however, much greater than described before, of six rats only two showed a slight increase after an intraperitoneal administration of 200 mg DL tryptophan. These results indicate clearly the importance of the intestinal flora for the nicotinamide saving action of tryptophan. If the tissues are concerned with this action at all, this can only be of secondary importance.

The fact that rising doses of tryptophan increase the nicotinamide formation, as indicated by the urinary elimination of its methyl derivative, up to a maximum which could not be raised by larger doses of tryptophan though it could be by nicotinamide application, indicated that a kind of tryptophan saturation has to be reached to obtain optimum nicotinamide formation. The maximum was reached in each of six rats of about 300 g when 200 mg of DL tryptophan were orally administered. If one were to assume an even distribution of the ingested tryptophan over the whole body, this would mean

that saturation was reached at a concentration of 3.3 mM. This assumption of even distribution is certainly incorrect and a comparison between rat and *E. coli* is not admissible. But it may be noted that in both rat and *E. coli* maximum nicotinamide formation was observed with tryptophan concentrations of the same order. The results with the rat also support the conception of a catalytic action of tryptophan in nicotinamide biosynthesis. However, there are some apparent objections to this conception. A catalytic action would mean that tryptophan would play the role of a coenzyme in the nicotinamide synthesis and the methyltryptophans substituted in the indole nucleus would, therefore, be metabolite antagonists to a coenzyme. No such case has been described so far. The concentration of tryptophan producing optimal nicotinamide synthesis compared with the amount of nicotinamide synthesized is high and thus is unusual for a catalyst. But catalytic reactions are known which require a fairly high concentration of the catalyst, e.g. nickel in catalytic hydrogenation or aluminium chloride in Friedel and Craft's reaction.

SUMMARY

1 The effect of tryptophan has been studied on the biosynthesis of nicotinamide by washed and broken *E. coli*, by undialysed and dialysed washings and washed fragments of the broken cells, in various salt solutions.

2 The effect of rising doses of tryptophan on the nicotinamide methochloride elimination by rats has been examined.

3 Nicotinamide was formed by washed and broken *E. coli* and by their undialysed and dialysed washings, but not by exhaustively washed cell fragments, in salt solutions containing sources of nitrogen other than tryptophan, such as ammonium ions or ornithine, the formation was increased by the addition of tryptophan.

4 No nicotinamide was formed by these cell preparations in the presence of tryptophan when no other source of nitrogen was present.

5 The effect of rising doses of tryptophan on the nicotinamide synthesis by washed *E. coli* reached a maximum at concentrations of 1–2 mM, a further increase of the concentration did not increase the synthesis of nicotinamide.

6 In rats comparative experiments on the effect of tryptophan administered either orally or intraperitoneally confirmed the importance of the intestinal flora for the action of tryptophan on the nicotinamide metabolism. By feeding rising doses of tryptophan it was shown that as in *E. coli* a saturation point could be reached.

7 The bearings of these findings on the possible role of tryptophan in the biosynthesis of nicotin

amide are discussed. It could best be explained by a kind of catalytic action of tryptophan on the nicotinamide synthesis.

NOTE ADDED 7 APRIL 1949

Since this paper was submitted, Heidelberger, Abraham & Lepkowsky (1948) have reported feeding DL tryptophan labelled in the β position of the side chain with ^{14}C to rats, nicotinamide methochloride eliminated after ingestion contained a labelled C atom in the carboxylic acid group. This does not, however,

prove, as is claimed by these authors, that 'the conversion proceeds in the same fashion in the rat as in *Neurospora*'.

This work was carried out with a grant for expenses from the Medical Research Council to one of the authors (P.E.). We wish to express our thanks to Dr A. Emmanuelowa for culturing and classifying the *E. coli* used and to Miss J. Piment, B.Sc., for disintegrating the *E. coli*, to Dr M. R. Pollock for valuable advice and to Dr G. P. Gladstone for reading and criticizing the typescript and to Miss A. Fullerton for technical assistance.

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Some Observations on the Amino-acid Distribution of Collagen, Elastin and Reticular Tissue from Different Sources

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(Received 6 April 1949)

By histological methods three types of connective tissue can be shown to be present in skins, collagen, elastic fibres and reticular tissue. Collagen, in the form of white bundles of interweaving fibres, makes up the greater part of the skin, the elastic fibres are pale yellow in colour and occur mainly in the grain layer, reticular tissue occurs around fat deposits and possibly as sheaths round the collagen fibre bundles (Dempsey, 1946). It is extremely difficult to isolate these three different tissues from skins. Tissues which stain similarly can be isolated fairly readily from other parts of the animal body: elastic fibres from the ligamentum nuchae (Vandegrift & Gies, 1901) and reticular tissue from lymph nodes (Bate Smith, 1947) and from the large fat deposits (Maximow & Bloom, 1935; Dempsey, 1946), and some studies have been made on them. Before these tissues can be assumed to be identical with those occurring in skin, however, more conclusive evidence than staining reactions is required.

The aim of the present investigation was to determine what major differences in composition existed between the three main types of connective tissue, and to obtain information as to whether similar tissue preparations from different sources had the same composition.

EXPERIMENTAL

Analytical methods

Total N, amide N, amino N and titration curves were determined as described by Bowes & Kenten (1948a).

Preparation of samples

Ox hide and alkali treated sheepskin collagen were prepared as previously described (Bowes & Kenten, 1948a, b). Reticular tissue was prepared from lymph nodes and from the adipose tissue of ox. The wet lymph nodes were sliced, extracted with 5% (w/v) NaCl, washed and macerated with many changes of 30% ethanol in water. The fibrous mass was

dehydrated in 98% ethanol, extracted with light petroleum at 35° and air dried. Sheets of tissue from the fat deposit of an ox were mechanically freed from fat, extracted with light petroleum at 35°, washed with water and ethanol and dried in air.

The total N of the reticular tissues was rather lower than that of the collagen or elastin. The amide N was higher than that of elastin or of alkali treated sheepskin collagen, and of the same order as ox hide collagen (Table 1).

Table 1 *Analyses of connective tissues*

(Moisture and ash free basis)

Preparation	Total N (%)	Amide N (mmol/g)	Amino N (mmol/g)
Collagen (ox hide)	18.6	0.06	0.46
Collagen (sheepskin, alkali-treated)	17.3	0.35	0.35
Elastin (ligamentum nuchae)	17.0	0.20	0.07
Reticular tissue (adipose tissue)	16.1	0.50	—
Reticular tissue (lymph nodes)	16.1	0.58	—

Elastin was prepared from the ligamentum nuchae of a freshly slaughtered ox. The tissue was freed from adhering matter, cut into approximately 0.5 cm cubes, extracted with 10% (w/v) NaCl, washed free from salt, dehydrated with acetone and extracted with light petroleum (b.p. 40–60°) in a Soxhlet for 12 hr. The air-dried material (180 g) was a pale yellow colour (N content 16.3%, moisture and ash free basis). To remove collagen, 168 g of this material were autoclaved with 500 ml water for 2 hr at 120°. The supernatant liquor and the hot-water washings were combined and the total N extracted determined (see Table 2). (Increasing

Table 2 *Soluble nitrogen extracted from ligamentum nuchae*

Extractions	Nitrogen extracted	
	(% dry tissue)	(% total N)
During autoclaving	2.00	14.92
Subsequent extractions with water		
First four	0.81	6.05
Fifth	0.066	0.49
Sixth	0.035	0.26
Seventh	0.008	0.06
Eighth	0.010	0.08
Total	4.921	21.86

the time of autoclaving to 3 hr caused no appreciable increase in the N extracted.) The autoclaved material was given eight further extractions of 1 hr duration with 350 ml portions of boiling water, and the total N removed in these extractions determined (Table 2). The elastin was dehydrated with acetone, again extracted with light petroleum and air dried (132 g). Assuming that the residual material is elastin, the ligamentum nuchae after extraction with salt and removal of fat contained about 78% elastin and 22% collagen. This is in agreement with the value of 81% elastin found by Vandegrift & Gies (1901) and later by Lowry, Gilligan & Katersky (1941) for elephant ligamentum nuchae.

The value for the total N of elastin (Table 1) is in agreement with previously determined values (Zoja (1897), 16.96, Richards & Gies (1902), 16.87, Stein & Miller (1938), 17.1%). The amide N, however, is appreciably lower than the two other values reported in the literature (Horbaczewski (1882), 0.41, Stein & Miller (1938), 0.38 mmol/g), this is probably due to the fact that under the conditions used in earlier methods N, other than amide N, was returned as NH₃.

RESULTS

Titration curve of elastin

The titration curve of elastin with hydrochloric acid in the presence of 0.5 M-sodium chloride is given in Fig. 1, together with the corresponding curve for ox-hide collagen. The elastin shows an isoelectric point in the neighbourhood of pH 6. The curve shows

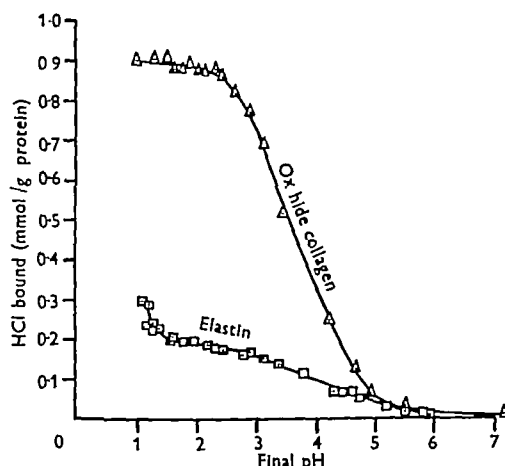


Fig. 1 Titration curves of elastin and ox-hide collagen in the presence of 0.5 M NaCl

a tendency to flatten between pH 2.0 and 1.5, but as the pH decreases further the amount of acid bound begins to increase again. This may be due to titration of fairly strong acid groups or of some weakly basic groups. The soluble nitrogen of the final solutions at pH values below 2.0 was only of the order of 0.5 mg/g elastin, indicating that there was little solubilization of the protein. The curve indicates the presence of at least 0.2 mmol basic groups per g elastin.

Paper partition chromatography of samples

The technique described by Consden, Gordon & Martin (1944) was followed. The air-dry tissue (10 ± 1 mg) was hydrolysed with 0.5–1.0 ml 6N HCl at 100° for 24–30 hr. The hydrolysate was concentrated three times *in vacuo*, dissolved in 0.2 ml water, and 10 μl applied to a large sheet of Whatman no. 1 filter paper. The chromatogram was run in one direction with *s*-collidine in an atmosphere of diethylamine and HCN, and in the other direction with phenol in an atmosphere of NH₃ and coal gas. The chromatograms were examined immediately after spraying with ninhydrin, and

an estimation of the amino acids present made from the size and colour intensity of the spots (Table 3). The strongest spots were arbitrarily assigned the value 9 and those just visible in a good light 1. Proline and hydroxyproline, which give yellow and orange colours respectively, were assessed as strong (S), medium (M) and weak (W).

Qualitative analyses by two dimensional paper chromatographs of the reticular membrane from fat deposits and of elastin from ligamentum nuchae have been reported (Jordan Lloyd, 1946). In general the results agree, except that Jordan Lloyd reports the presence of hydroxyproline in elastin, this may be due to incomplete removal of the collagen. This author also reports that reticular tissue from fat deposits contains no tyrosine or hydroxylysine, whereas these were present in small amounts in our preparations. Consideration of the figures in Table 3 shows that elastin differs from collagen and both

reticular tissue is converted to gelatin and a loose powdery substance which he terms 'reticulum' and which only slowly passes into solution. Both tissues lost approximately 50% in weight, but while the chromatogram of the lymph-node tissue was not appreciably changed, that of the reticular tissue from the fat deposits showed a definite decrease in hydroxyproline. This suggests that this reticular tissue contained some collagen, and, in confirmation, the aqueous extract obtained on autoclaving formed a gel, while a similar extract from lymph nodes did not (see also Bate Smith, 1947). On autoclaving for 18 hr at 105°, 73 mg of the air-dry reticular tissue from the fat deposits gave a residue of only 6 mg. This residue resistant to autoclaving might be expected to be elastin, but the paper chromatogram showed that this was unlikely since it contained appreciably more of the di-

Table 3 *The amino-acid distribution in some preparations of connective tissues as indicated by the relative intensities of spots on paper chromatograms*

Material Source Amino-acids	Collagen			Reticular tissues					Collagen residues	
	Alkali treated sheep skin		Elastin Ligamentum nuchae	Lymph nodes		Fat deposits			Ox hide	Alkali treated sheep skin
	Ox hide	sheep skin		Macerated	Auto claved 4 hr	Extracted with light petroleum	Auto claved 3 hr	Auto claved 18 hr		
Alanine	7	7	9	8	8	9	8	8	6	9
Glycine	9	9	9	9	8	9	9	8	6	9
Leucine, etc *	5	6	9	9	9	7	7	9	9	8
Phenylalanine	2	2	3	3	3	2	2	2	3	3
Valine	5	5	8	5	5	6	6	7	7	7
Cysteic acid	1?	1?	1	1	1	1	1	1	?	1
Serine	3	3	2	5	4	5	4	4	4	4
Threonine	2	2	2	4	4	3	3	4	3	3
Tyrosine	1	1	2	3	3	1	2	1	2	2
Arginine	5	5	1	6	6	4	6	3	4	3
Histidine	2	2	—	2	2	1	1?	—	2	1
Hydroxylysine	2	2	—	1	1	2	2	—	—	—
Lysine	5	5	1	6	7	4	5	5	5	3
Aspartic acid	5	6	3	7	7	5	6	5	7	4
Glutamic acid	7	7	4	8	8	7	9	6	8	6
Proline	S	S	S	M	M	M	M	M	M	M
Hydroxyproline	S	S	—	M	M	M	W	Trace	—	—

* Leucine, etc., refers to a composite spot which may contain leucine, isoleucine, and methionine

types of reticular tissue in containing less arginine, lysine, hydroxyproline and glutamic acid, rather less aspartic acid, and rather more valine. In addition, it differs from the reticular tissues in containing rather less serine and rather more proline. Collagen differs from the reticular tissues in containing more proline and hydroxyproline. The reticular tissue from the fat deposits contains rather less of the dicarboxylic and basic amino acids than the lymph node tissue.

It was considered possible that the reticular tissues were contaminated with collagen. The tissues were autoclaved for 3 hr at 105°. This should remove collagen, but according to Siegfried (1902)

carboxylic and basic amino acids than elastin from ligamentum nuchae. It is possible that it is identical with Siegfried's 'reticulum'. Similarly, the residues (11 mg) obtained on twice autoclaving ox hide (2 g) for 6 hr at 105° followed by boiling for 1-2 hr, and the residue (30 mg) obtained by boiling 4.76 g dried sheepskin with 100 ml 0.01 N sodium hydroxide for 15 min, followed by repeated extractions with boiling water, gave paper chromatograms distinguishable from that of elastin by the presence of histidine and larger amounts of the dicarboxylic and basic amino acids. The two residues differed slightly from one another, particularly in respect to the dicarboxylic acids, and from lymph node reticular

tissue in containing no hydroxyproline. The absence of this amino acid indicated the complete removal of collagen.

DISCUSSION

It is of interest to compare values given in the literature for the amino acid composition of elastin

Table 4 *Composition of elastin*

	Amino acid		Relative intensities of spots on paper chromatograms
	(g/100 g)	(N as % protein N)	
Glycine	29.4 (a) 25.75 (b)	32.3 28.3	9
Alanine	6.85 (b)	6.3	9
Leucine fraction	30.0 (a) 21.38 (b) 10.7 (h)	18.8 13.4 6.7	9*
Valine	13.5 (a) 13.8 (h)	9.5 9.7	8
Phenylalanine	3.89 (b) 3.34 (c) 4.8 (h)	1.9 1.7 2.4	3
Tryptophan	0.0 (a)	—	—
Serine	—	—	2
Threonine	2.7 (g) 1.1 (h)	1.9 0.8	2
Cystine	0.23 (a) 0.6 (h)	0.2 0.4	(Cysteic acid = 1)
Methionine	0.38 (a) 0.03 (h)	0.2 —	—
Proline	15.2 (a) 15.6 (h)	10.9 11.2	5
Hydroxyproline	2.0 (a)	1.3	0
Arginine	1.0 (a) 0.3 (f) 1.1 (h)	1.9 0.6 2.1	1
Histidine	0.0 (a) 0.04 (h)	0.0 0.1	0
Lysine	0.0 (a) 0.5 (h)	0.0 0.6	1
Hydroxylysine	—	—	0
Aspartic acid	0.0 (a) 0.6 (b)	0.0 0.4	3
Glutamic acid	2.7 (b) 3.3 (h)	1.5 1.8	4
Tyrosine	1.6 (b) 0.25 (d) 0.34 (e) 1.4 (h)	0.7 0.1 0.2 0.6	2

* Isoleucine is present

(a) Stein & Miller (1938) (b) Abderhalden & Schittenhelm (1904) (c) Kapeller Adler (1932) (d) Horbaczewski (1882) (e) Schwarz (1893) (f) Kossel & Kutscher (1898) (g) Brand & Kassel (1942) (h) Graham *et al.* (1949)

with the results of paper partition chromatographic examination of the present preparation of elastin (Table 4). The chromatogram indicates that the values reported for aspartic acid and alanine are

low, and that serine is present in approximately the same amount as threonine. The low figures for leucine and isoleucine obtained by Graham, Waitkoff & Hier (1949), using microbiological methods, suggest that the 'leucine fraction' of Table 4 may contain amino acids other than leucine and isoleucine in comparatively large amounts. No hydroxyproline was detected in the chromatogram of the present preparation, and although it may not be entirely absent, the value of 2% given by Stein & Miller (1938) is possibly too high and may be due to incomplete removal of collagen. The low value for the amino nitrogen is in agreement with the small amount of lysine detected by analysis and by paper chromatography. The titration curve indicates that at least 0.2 mmol/g of basic groups must be present in elastin, the amino nitrogen (0.07 mmol/g), together with 0.06 mmol/g of arginine (Stein & Miller, 1938), accounts for 0.13 mmol/g, leaving 0.07 mmol of base to be accounted for. Histidine was not detected by paper chromatography, and consequently is present, if at all, in very small amount, it seems likely, therefore, that the discrepancy between the titration curve and analysis is due to a low arginine value.

Taking the highest figure for the 'leucine fraction' (30%) it is possible to account for approximately 90% of the total nitrogen of elastin. Judging from the chromatogram and the titration curve, about half this deficiency is due to the presence of more serine, arginine and aspartic acid than the present analytical figures suggest, leaving 5% still to be accounted for. It may be noted that Stein & Miller (1938) find that the sum of sulphur in methionine (0.08%), cystine (0.06%) and occurring as SO_4^{2-} (0.02%) agrees closely with the total sulphur of elastin (0.16%).

Comparison of the chromatograms suggests that elastin and reticular tissue from different sources may differ appreciably in composition. The residues from the exhaustive extraction of ox-hide, sheepskin and reticular tissue from fat deposits, which on the reported properties of collagen, elastin and reticular tissue, should consist mainly of elastin, do not give chromatograms similar to that of elastin from ligamentum nuchae, but, with the exception of the absence of hydroxyproline, give chromatograms more nearly resembling that of lymph node reticular tissue. It appears, therefore, that the elastic fibres of skin are either appreciably different in composition from those of the ligamentum nuchae, or that they are less resistant to boiling water, and the residues obtained from skin consist of some other protein or proteins resembling reticular tissue in composition.

In view of the suggestions that have been made regarding the possible identity of reticulin and pre-collagen (Heringa & Weidinger, 1942) it is of interest

to note that with the exception of a lower proline and hydroxyproline content, the amino acid distribution of the collagen and reticular tissue preparations are similar

SUMMARY

1 Collagen has been prepared from ox hide, elastin from ligamentum nuchae and reticular tissue from lymph nodes and fat deposits. Total nitrogen, and amide nitrogen have been determined and paper chromatograms of the hydrolysates examined.

2 Examination of the titration curve and chromatogram of elastin indicates that several amino acids have still to be determined and that the reported values for arginine and alanine are low.

3 Tissues from different sources which stain similarly may vary appreciably in composition.

With the exception of a lower proline and hydroxyproline content, the amino acid distribution of the reticular tissues was similar to that of collagen.

4 Examination of chromatograms of the residues obtained by the exhaustive extraction of skin suggests that the elastic fibres of skin either differ from those of ligamentum nuchae with respect to amino acid composition or are less resistant to hot water.

The authors wish to thank the Director of the Wool Industries Research Association, in whose laboratories the paper partition chromatographic analysis was carried out, for the facilities put at their disposal, and Drs R. Consden, A. H. Gordon and A. J. P. Martin for much useful advice and assistance. Thanks are also due to the Council of the British Leather Manufacturers' Research Association for permission to publish this paper.

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A Differential Method for the Detection of Small Differences in Mobility of Colloids in Electrophoresis

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(Received 24 January 1949)

The determination of differences between the mobilities of two colloids by the standard technique of measuring the boundary displacements in two separate experiments necessitates the rigorous prevention or control of movements of the boundaries due to causes other than the migration of the colloid in the electric field. The method described here has the advantage of eliminating possible complications of that kind. Its main feature is the simultaneous observation of the boundaries of two components A and B, in an electrophoresis run, in which a layer

of a solution of one component, A or B, is put on top of the mixture of A and B, the concentration of A or B being the same in both layers.

Two experiments are set up as shown in Fig. 1a, b. The boundaries are first brought into view (Fig. 1c) and, after the current has been started, are kept in position by compensation, i.e. by running buffer into the electrode vessel towards which the migration takes place. The distance between the two ascending boundaries is recorded at intervals. Experiments have also been made in which the supernatant liquid

on either side of the mixture is a solution of one of the components. In this case the experiment was set up as shown in Fig 1d, e

solution. A gravitationally unstable region necessarily forms at the boundary. Judging by the shape of the boundaries in experiments in which diluted solutions were prepared from

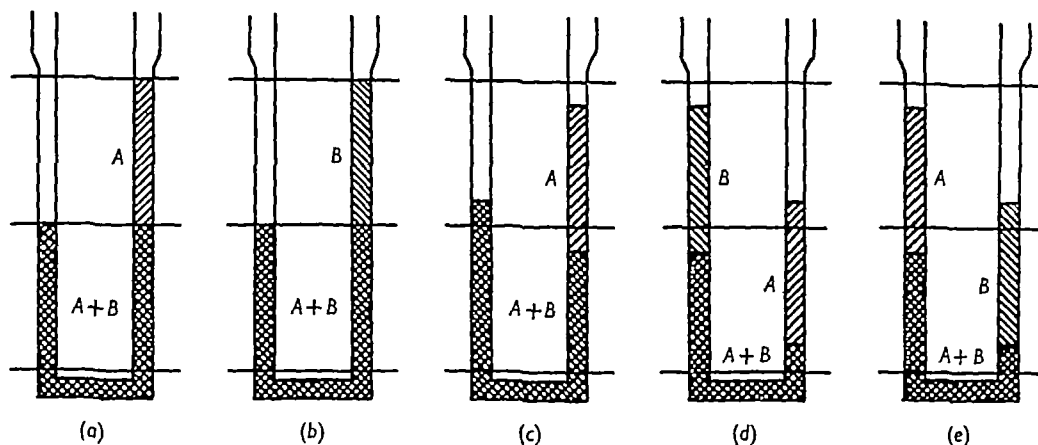


Fig 1 Arrangement of solutions in electrophoresis cell. Ascending, right; descending, left

THEORETICAL

In the following discussion colloids only are considered which give gravitationally stable boundaries in ordinary runs in the same buffer. Two kinds of interference may arise. (a) When solutions equilibrated by dialysis are used, δ boundaries appear below both the lower and the upper ascending boundaries. The velocity of migration of the lower ascending boundary is bound to change when this boundary and the δ boundary of the solution in the upper layer cross. The δ boundaries can be almost completely eliminated if the solutions containing A or B are prepared from those which establish themselves, on preliminary electrophoresis of A and B respectively, below the ascending boundary ('adjusted solutions', Longworth & MacInnes, 1940a, Svensson, 1946). If the same buffer is used as supernatant throughout, A solution prepared by dilution of an adjusted solution with the buffer is not identical with one recoverable from below the ascending boundary after an electrophoresis in which the colloid concentration had been the same as that of the diluted solution. One may expect the differences in the ionic concentrations between these two solutions to be small, and the δ boundaries negligible, if the change in migration velocity of the ascending boundary is approximately proportional to the change in colloid concentration, within the concentration range between the undiluted and diluted solutions. This range is greatest at low concentrations and it increases with the salt concentration of the buffer solution.

In an experiment as in Fig 1a, b and c with 'adjusted solutions', the pattern on the descending side represents the electrophoresis of the mixture of the two components in which the ϵ boundary is absent. Usually the conductivity of the 'adjusted solution' is less than that of the buffer solution, and the migration velocity of the colloid, the current being kept constant, increases with increasing colloid concentration. This increase is greater at higher colloid concentration since the transport number of the colloid also increases. Thus on dilution of an 'adjusted solution' with buffer the migration velocity of the colloid will be slightly greater than in a solution adjusted at the concentration of the diluted

'adjusted solutions' containing 1-1.5% protein, this boundary disturbance appears to be negligible for albumin in phosphate at $\mu=0.1$ and oxyhaemoglobin (HbO_2) in phosphate at $\mu=0.05$ (μ =ionic strength).

(b) According to the moving boundary equation (Svensson, 1943, 1946, Dole, 1945) the concentrations of all ions change at every boundary. Since in the present experiments the colloid concentration in the upper compartment was chosen to be the same as in the mixture in the bottom compartments, 'false' boundaries (as defined by Svensson, 1946, p. 14, i.e. boundaries where no ion disappears) due to changes in concentration of that component which is present on both sides of the lower ascending boundary, must be expected ahead of this boundary if the faster, and behind it if the slower of the two components is placed above the mixture. In at least one of these cases a gravitationally unstable region is bound to form at the lower ascending boundary. Thus, if the faster component A is placed above the mixture of A and B, the boundaries will be gravitationally stable if $V_{Am} > V_A$ (V_A =migration velocity of A in unitary solution, such as in the solution between boundaries 1 and 1* of Fig 2a, V_{Am} =migration velocity of A in the mixture), and a false boundary, 1*, will form ahead of 2 (Fig 2a). If $V_{Am} < V_A$ the boundary region above 2 is gravitationally unstable and it will rise to a level above 1 (Fig 2b, c). In both cases the change in the distance between the ascending boundary regions of A and B is less than it would be if no changes occurred in the concentration of A at the boundary of B. When B is placed above the mixture and if $V_{Bm} > V_B$ (V_B =migration velocity of B in unitary solution, V_{Bm} =migration velocity of B in the mixture), a gravitationally unstable region is probable near the boundary of A. It is conceivable that the material in this region flows off as soon as it separates, and that the migration velocity of the boundary of A is equal to V_{Am} . If $V_{Bm} < V_B$ (this is only likely if $V_{Am} < V_A$ and not if $V_{Am} > V_A$, since interaction, in the sense of specific attraction, between A and B would make the average migration velocities differ less, but not more), a false boundary will form below 2, and the change in the distance between the boundaries of A and B will also

appear reduced here (Fig 2d) It is mainly because of these boundary anomalies that the experiments must be carried out at low colloid concentrations It does not seem possible to derive the upper limit of colloid concentrations permissible by calculation without making assumptions about the mobilities of the components in the mixture This limit certainly depends on the relative difference in mobility of the two components

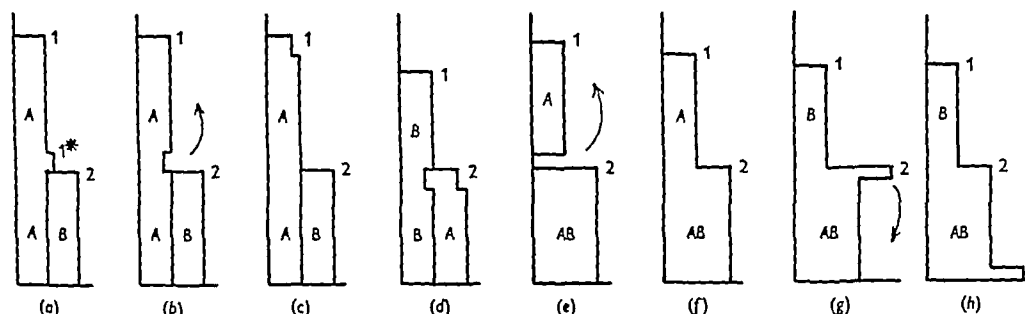


Fig 2 False boundaries in the differential method Abscissae = concentrations of colloid, ordinates = vertical distances in electrophoresis cell

Assuming that V_{Am}/V_A does not differ appreciably from unity and disregarding, for the present argument, the boundary disturbances discussed above, the difference of the changes after unit time in the distances between the two ascending boundaries, in experiments as in Fig 1a, b, is equal to the sum of the absolute migrations, and is given by

$$d = (V_A - V_{Bm}) - (V_B - V_{Am})$$

Introducing ϵ by $\frac{V_{Am} - V_{Bm}}{V_{Am}} = \frac{V_A - V_B}{V_A} (1 - \epsilon)$,

$$d = V_A - V_B + \frac{V_{Am}}{V_A} (V_A - V_B) (1 - \epsilon),$$

and the difference in migration velocity of A and B

$$V_A - V_B = \frac{d}{1 + \frac{V_{Am}}{V_A} (1 - \epsilon)}$$

If $V_B/V_A = V_{Bm}/V_{Am}$, which is probable if no interaction occurs between the two components, and if the colloid concentration is sufficiently low for the differences between the effects of ionic strength on the mobility of each component to be negligible, then

$$\epsilon = 0 \text{ and } V_A - V_B = \frac{d}{1 + \frac{V_{Am}}{V_A}} \approx \frac{d}{2},$$

if $V_{Am} - V_{Bm} < V_A - V_B$, indicating interaction between the components in the mixture, then $\epsilon > 0$ and $V_A - V_B > \frac{1}{2}d$. If $V_{Am} - V_{Bm} = 0$, i.e. maximum interaction, the mixture of the two components would behave as one component and gravitationally unstable regions would form near the lower of the two boundaries. Imagining the transfer of the unstable regions to stable positions to occur without mixing with the liquid through which they pass, the events may schematically be represented as in Fig 2e-h. On migration, with the faster component in the upper layer, the distance between the ascending boundaries would not change, while with the slower component in the upper layer the change in the distance in unit time would be equal to the difference

in migration velocity between the slower component and the complex, i.e.

$$d = V_B - V_{Am} = \frac{1}{2} (V_B - V_A) \text{ if } V_{Am} = V_{Bm} = \frac{1}{2} (V_A + V_B)$$

In an actual experiment the gravitationally unstable region would mix, at least partially, consequently $d > \frac{1}{2} (V_B - V_A)$

It can be concluded that at infinitely low colloid concentration, and in the absence of interaction between the

two components, the difference between the velocities of the two components is very nearly equal to the change per unit time in the distance between the two ascending boundaries. In this case V_{Am}/V_A involves only the ratio of cross sections at the levels of the boundaries. At finite colloid concentrations the difference in migration velocity of the components is always greater than this, as shown by the analysis of the possible combinations of relative values for V_A , V_{Am} , V_B and V_{Bm} .

Thus a limitation of the method is indicated on theoretical grounds by the finding that, on account of boundary anomalies and possible interaction between the components, a minimum value only for the difference in mobility can be obtained. The difference in mobility, $u_A - u_B$, corrected to infinitely low colloid concentration (Hoch, 1948b), is given by

$$u_A - u_B = \frac{\kappa q}{I} \left(\frac{V_A}{1 + K_A C_A} - \frac{V_B}{1 + K_B C_B} \right),$$

where κ = conductivity of the buffer solution, q = area of cross section at the position of the upper boundary, I = current, K_A and K_B = coefficients defined by $K_i = \frac{V_{ai} - V_i}{C_i V_i}$ (V_{ai} = actual migration velocity of ascending boundary, V_i = migration velocity of component i at infinitely low concentration in the supernatant buffer, C_i = concentration of component i in g/100 ml). If the concentrations C_A and C_B are chosen so that $K_A C_A$ and $K_B C_B$ are equal, then

$$(u_A - u_B) = \frac{\kappa q}{I (1 + K_A C_A)} (V_A - V_B)$$

Substituting for $(V_A - V_B)$,

$$(u_A - u_B)_{\min.} = \frac{\kappa q}{I (1 + K_A C_A)} \frac{d}{1 + \frac{V_{Am}}{V_A} (1 - \epsilon)} \quad (1)$$

Although an estimate of possible deviations of the expression for $(u_A - u_B)_{\min.}$ from the true difference in mobility cannot be made, a qualitative argument may provide a means of

limiting the range of uncertainty. It can be shown (Hoch, 1948a) that the boundary anomalies become considerable when the relative change in migration velocity of an ion across a protein boundary is of the same magnitude as the relative difference in the mobilities of that ion and of the protein. If, for example, the mobilities differ by five times this amount, the concentration changes across the protein boundary may be disregarded, and the uncertainty in the difference in mobility as calculated from equation (1) can be limited to the effects of interaction. Thus, for small relative differences in mobility, about as great as the relative change in migration velocity of ions across the protein boundary, a value of five times that obtained by equation (1) may arbitrarily be taken as the probable upper limit for the true value, assuming little or no interaction. With comparatively large relative differences in mobility, the boundary anomalies may be neglected. Favourable working conditions are therefore near the isoelectric point and at high salt concentrations, so that the value for the difference in mobility

may be bracketed between $\frac{\kappa q}{I} \frac{d}{2}$ and $\frac{\kappa q}{I} 2d$ if there is interaction between the components. In view of these uncertainties the following approximate form of equation (1) was used in the present paper

$$(u_A - u_B)_{\text{min.}} = \frac{\kappa_B q}{I} \frac{d}{2}, \quad (2)$$

where κ_B = conductivity of the buffer solution

The method appears to be useful for the purpose of establishing the identity of two colloids. Here K_A and K_B are assumed equal and experiments (a) and (b) of Fig. 1 must give identical changes in the relative positions of the boundaries if $C_A = C_B$. However, identical changes might be obtained also if $\frac{V_A}{1 + K_A C_A} = \frac{V_B}{1 + K_B C_B}$ for $V_A \neq V_B$ at a particular concentration of A (=that of B). But the uncertainty should be resolvable on carrying out the experiment at different salt or colloid concentrations. For example, if $K_A = 0.06$ and $K_B = 0.05$, $C_A = C_B = 0.3$ g./100 ml. V_A/V_B could be 1.003, although no difference would be found. But at $C_A = C_B = 0.06$ g./100 ml. a relative difference of 0.3% in the migration velocities should be detected.

The differential method was applied to the comparison of serum and urine albumins, and to that of human and rabbit oxyhaemoglobin.

EXPERIMENTAL

The experiments were made in the Tiselius apparatus at 0.1°. Three different medium-size (11 ml.) cells with two middle sections and the cylindrical lens schlieren optical system (Thovert, 1914; Philpot, 1938; Svensson, 1939) were used.

Materials. Fresh blood and urine were obtained from nephritis patients with albuminuria. The solutions of HbO_2 were prepared as follows. 1–2 ml. of packed red cells were washed 6–8 times with 2 vol. of a mixture consisting of 0.85% NaCl (2 vol.) and Na phosphate buffer, pH 8, μ , 0.2 (1 vol.). The cells were then lysed with 2 vol. of water and the solution centrifuged. On addition of pH 8 phosphate buffer to the clear supernatant a precipitate formed which was centrifuged down. This precipitation eliminated the fast component and the slight turbidity which was previously

observed by Stern, Reiner & Silber (1945). The supernatant liquid was clear in every preparation.

Solutions of the sera were made up in Na phosphate buffers, pH 7.7 or 8.0, and ionic strength 0.1, those of HbO_2 in Na phosphate buffers, pH 7.1 and 8.0. The pH 7.1 buffer was that used by Andersch, Wilson & Menten (1944). The ionic strength of the pH 8 buffer used for the electrophoresis of HbO_2 was 0.05.

The adjusted solutions of albumin were prepared by the electrophoresis of serum at concentrations yielding albumin solutions containing 0.4–1.5 g. protein/100 ml. The HbO_2 solutions were electrolysed at concentrations of about 1 g./100 ml. The preparatory runs were continued until two thirds or the whole ascending limb was occupied by the adjusted solution. The adjusted solutions were appropriately diluted with buffer and equal volumes of each component, A and B , were mixed to give the solution for the bottom layer, smaller portions were diluted with an equal volume of buffer to give the solutions for the upper layer.

The concentrations of albumin and of HbO_2 were determined by micro Kjeldahl. HbO_2 was also determined by measuring the light absorption of HbO_2 and of HbCO in dilute NH_3 (2.5 ml. of NH_3 sp. gr. 0.880/100 ml.) in the visible region.

Observation of boundaries. The boundaries were brought into view by compensation and the patterns, which were obtained with a diagonal wire in all differential experiments, were photographed at frequent intervals during the run. The use of a wire (Svensson, 1939) facilitates the location of the line forming the pattern and this pattern is not subject to distortion if unevenly exposed (Svensson, 1939). The enlarged patterns were traced on graph paper, and the position of the centroidal ordinates of the peaks (Longworth, 1943) was determined by making fifteen to thirty readings, spaced at equal distances, on each boundary. The following form of the equation for the centroidal ordinate (\bar{x}) was found convenient for use without a calculating machine

$$\bar{x} = \frac{\sum xy}{\sum y} = \frac{\sum (y_i - y_{-i}) |x_i|}{2 \sum y_i - \sum (y_i - y_{-i}) + y_0},$$

where y_i and y_{-i} = ordinates at symmetrical positions on both sides of a working zero near the expected position of the centroidal ordinate, y_0 = ordinate at the working zero, x_i = abscissae.

RESULTS

(a) **Albumin.** Table 1 gives the details of experiments with serum and urine albumins in three cases of nephritis, and Fig. 3 shows the plot of the distance between the ascending boundaries against time in cases 1 and 3. The minimum difference in mobility calculated from equation (2) was about 0.03×10^{-5} , i.e. 0.5% of the mobility of albumin, in two cases. In case 3 the difference in mobility was much greater, but the urine had been concentrated to a quarter of its volume before dialysis and this may have altered the mobility of the urine albumin.

It appears from Fig. 3 that it might be possible to detect an even smaller difference in migration velocity of two albumin boundaries than that found in case 1. The interpretation of such a small difference is difficult, apart from the interference by the

Table 1 Comparison of electrophoretic mobilities of serum and urine albumins

Case no	(1*)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
1	6.0	30	1.0	8.0	U 0.11 S 0.11	6.3	0.053 (± 0.02)	400	0.2	0.03	U
2	5.6	123	0.5	7.7	U 0.18† S 0.22	5.7	0.04 (± 0.04)	400	0.2	0.03	U
3	7.0	200	0.1	7.7	U 0.16 S 0.17	5.7	0.76 (± 0.04)	520	2.4	0.42	U

* Column designation

(1) Serum proteins, g/100 ml.

(2) Non protein nitrogen, mg/100 ml

(3) Urine protein, g/100 ml

(4) pH of phosphate buffer, $\mu=0.1$

(5) Albumin concentration in upper layer, g/100 ml, U=urine albumin, S=serum albumin

(6) Potential gradient, V cm⁻¹

(7) Total change in distance between ascending boundaries, cm (approximate error range)

(8) Total time, min

(9) Difference in migration velocity of boundaries, 10⁻⁵ cm sec⁻¹(10) Minimum difference in mobility of components, from equation (2), 10⁻⁵ cm² V⁻¹ sec⁻¹

(11) Faster component

† Concentrations estimated from refractive areas in this case only

boundary anomalies discussed above, since it could also be produced by a change in pH of about 0.02 unit in one of the solutions

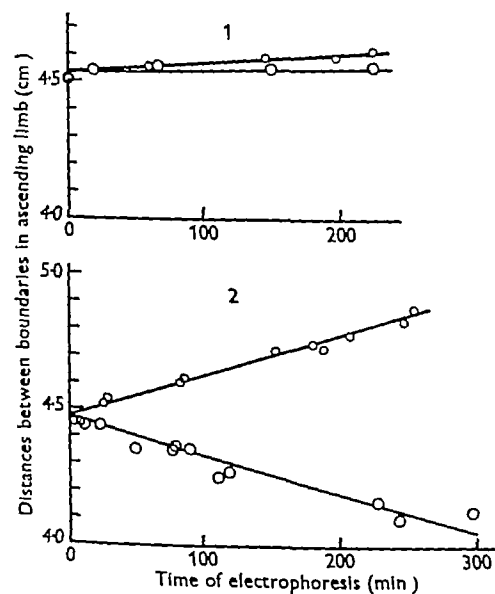


Fig 3 Diagrams obtained by the differential method albumins smaller circles for the runs with the urine albumin in the upper layer 1=case no 1 of Table 1, phosphate buffer, pH 8, $\mu=0.1$, 6.3 V/cm 2=case no 3, phosphate buffer, pH 7.7, $\mu=0.1$, 5.7 V/cm

In the three cases studied, the mean mobility of the urine albumin was the same or greater than that of the serum albumin from the same patient. It is necessary to speak of mean mobilities only, since both the serum and the urine albumin peaks proved composite in the preliminary runs as shown in Fig 4. Serum albumin has previously been shown to be

inhomogeneous at pH 8 (Blix, Tiselius & Svensson, 1941, Hoch & Morris, 1945, Armstrong, Budka & Morrison, 1947, Hoch, 1948b).

(b) *Haemoglobin* In Table 2 and Fig 5 are recorded the data of the comparison of the mobilities of oxyhaemoglobins from four different rabbits with the oxyhaemoglobin from a normal human adult. Rabbit oxyhaemoglobin had a smaller anodic mobility in all cases. There was no evidence of a difference between any of the normal rabbit oxyhaemoglobins.

Fig 6 shows the patterns obtained in Exp 3 of Table 2. The slowness of migration at pH 7.1 did not make the preparation of adjusted solutions practicable, and solutions equilibrated by dialysis were used. This was thought justifiable on account of the high ionic strength of the buffer and the small charge of the oxyhaemoglobin near its isoelectric point. The experiment was made at comparatively high protein concentration (0.5 g/100 ml) so that boundary disturbances could be more readily observed. In both experiments set up as in Fig 1d, e, the boundaries of the solutions of the single components did not appear to be disturbed by the formation of gravitationally unstable regions, but the shape of the curves deviated markedly from that of the Gaussian error curve. In the absence of boundary anomalies the lines relating the changes with time of the distances between the two ascending and the two descending boundaries should run parallel. That they diverged slightly (Fig 5, no 2) may have been due to the effects of the boundary anomalies.

Fig 7 shows some patterns from Exp 2 of Table 2, made at an unusually low protein concentration of 0.05 g/100 ml. The graph of the distances between the boundaries is seen in Fig 5, no 1.

If identity of two substances is suspected, the experiment may be carried out with solutions which

Table 2 Comparison of electrophoretic mobilities of oxyhaemoglobins

Exp no	(1)*	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	8.0	H 0.66 R 0.69	14	Asc 1.01	300	5.6	0.4	H
2	8.0	H 0.05 R 0.05	10	Asc 0.49 Desc 0.49	220 220	3.7 3.7	0.37	H
3†	7.1	H 0.5 R 0.5	8.7	Asc 1.02 Desc 1.04	600 600	2.83 2.89	0.33	H
4†	8.0	H _(A) 0.47 H _(B) 0.47	14	Asc 0.00	280	0	0	—

* Column designation

(1) pH of phosphate buffer, $\mu=0.05$ at pH 8, $\mu=0.08$ at pH 7.1(2) Oxyhaemoglobin concentration in upper layer, g/100 ml, H=human HbO₂, R=rabbit HbO₂, H_(A) and H_(B)=oxyhaemoglobins from two normal subjects(3) Potential gradient, V cm⁻¹

(4) Total change in distance between the two ascending or the two descending boundaries, cm

(5) Total time, min.

(6) Difference in migration velocity of boundaries, 10⁻⁵ cm sec⁻¹(7) Minimum difference in mobility of components, from equation (2), 10⁻⁵ cm² V⁻¹ sec⁻¹

(8) Faster component (anodic)

† In these experiments solutions equilibrated by dialysis were used

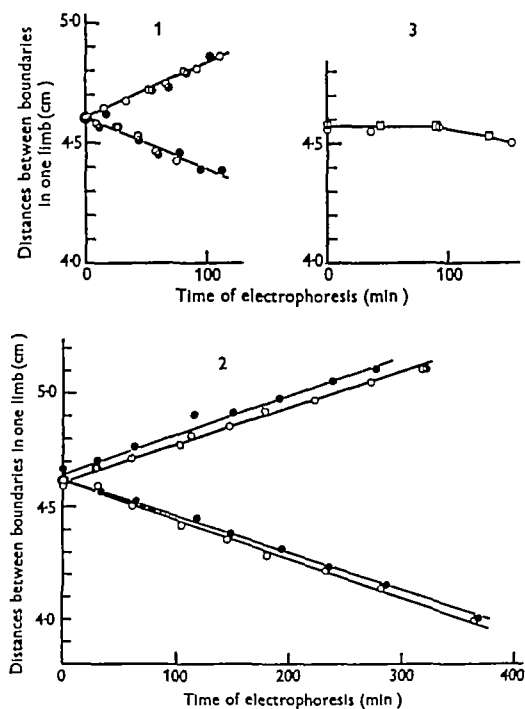


Fig 5 Diagrams obtained by the differential method oxyhaemoglobins Exp nos 2-4 of Table 2. Hollow circles for the ascending, full circles for the descending limb, circles and squares in Exp 4 for the ascending limb, with HbO₂ from subjects A and B, respectively, in the upper layer. The rising lines represent experiments in which the human, the falling lines those in which the rabbit HbO₂ was in the upper layer of the ascending limb. 1=Exp no 2 phosphate buffer, pH 8, μ , 0.05, 10 V/cm, HbO₂ concentration in upper layer, 0.05 g/100 ml. 2=Exp no 3, phosphate, pH 7.1, μ , 0.08, 8.7 V/cm, 0.5 g HbO₂/100 ml. 3=Exp no 4 phosphate buffer, pH 8, μ , 0.05, 14 V/cm, 0.47 g HbO₂/100 ml.

have not been subjected to preliminary runs for the preparation of adjusted solutions. Such an experiment is illustrated in Fig 5, no 3. The two adult human oxyhaemoglobins used were equilibrated simultaneously by dialysis against the same buffer solution. The migration was stopped before the δ boundary of the solution, originally in the top compartment, reached the lower boundary. No difference was found in the relative positions of the two ascending boundaries at the end of the run, between Exp (a) in which the oxyhaemoglobin from subject A, and Exp (b) in which the oxyhaemoglobin from subject B, was placed in the top compartment.

The tests for homogeneity of the dialysed oxyhaemoglobin preparations are summarized in Table 3. The experiments on human oxyhaemoglobin showed a component of smaller anodic mobility apart from the main peak in every case. At pH 7.1 no δ or ϵ boundary was seen to separate, but these boundaries may have been covered by the small component. The preparations of rabbit oxyhaemoglobin did not show this component (Table 3, column 1, Fig 8, nos 1 and 2). The ratios N/E^{HbCO} of different preparations were reproducible to within $\pm 2.5\%$, but those of human and rabbit carboxyhaemoglobin (HbCO) differed significantly (column 2). So did the ratios E^{HbCO}/E^{HbO_2} (column 3).

(c) *Mixtures of human and rabbit oxyhaemoglobins*
 These experiments were made with the object of obtaining an independent estimate of the difference between the migration velocity of the faster component, in unitary solution, and that of the slower component in the mixture at low oxyhaemoglobin concentrations, and to study the effects of boundary anomalies at higher concentrations. The results are given in Table 4 and Fig 8. The concentrations of human and rabbit oxyhaemoglobin were made equal

Table 3 *Tests for homogeneity of oxyhaemoglobin preparations*

Red cells from	(1)	(2)		(3)	
	Electrophoresis refractive area of slow component (% of total area)	$N \times 5.95/E_{604}^{HbCO*}$		$E_{604}^{HbCO}/E_{604}^{HbO_2}$	
		Mean (no of samples)	Range	Mean (no of samples)	Range
Human (three subjects)	3-5.5 (5 prep)	1.018 (4)	0.985-1.036	1.225 (6)	1.214-1.234
Rabbit (three animals)	0.5 (1 prep) or none detected (2 prep)	1.050 (4)	1.029-1.065	1.209 (8)	1.200-1.217

* N content of horse HbCO = 16.8% (Chibnall, Rees & Williams, 1943) The conversion factor for the colorimetric estimation was obtained by standardization against spectrographic data (Hoch & Jope, 1947, unpublished experiments)

Table 4 *Electrophoresis of mixtures of human and rabbit oxyhaemoglobins of known composition*

Exp no	Concentration of total HbO ₂ in adjusted solution (g/100 ml.)	Proportion of human HbO ₂ in terms of N content		Difference in mobility* of components (10 ⁻⁵ cm ² V ⁻¹ sec ⁻¹)	
		Taken (%)	Found (%)		
1	0.5	48.4	30	0.28	—
2	0.53	48.9	35	0.23	Bad separation
3	0.6	48.7	35	0.31	—
4	0.67	48.9	45	0.29	—
5	0.89	48.9	45	0.20	Bad separation
6	1.1	48.9	50	0.24	—
7	1.87	48.3	60	0.25	—

* Mobility calculated using potential gradient in supernatant buffer

with respect to the light absorption of the carboxyhaemoglobin, using the Ilford spectrum green filter no. 604. Assuming the refractive areas to be proportional to the nitrogen content, the proportion of the area under the faster of the two main peaks should be about 49% of the total area under the two main peaks, after allowing for the presence of 5% of the more slowly migrating component in the preparations of human oxyhaemoglobin, and for the difference in the contents of N/unit extinction (Table 3). The increase in concentration in the direction of migration of the human oxyhaemoglobin across the boundary of the rabbit oxyhaemoglobin in the buffer used was calculated to be appreciable at total concentrations of 0.5 g/100 ml. or greater, using assumptions about the similarity of the two components of oxyhaemoglobin such as specified for two albumins previously (Hoch, 1948b), and a value for $K = 0.1 (\pm 0.2)$ for both oxyhaemoglobins. This value has been obtained from experiments analogous to that previously described for serum albumin in which layers of concentrated and diluted solutions were made to migrate simultaneously (Hoch, 1948a). As can be seen in Fig. 8 and Table 4, the ascending patterns did not show the expected excess of the faster component (human oxyhaemoglobin). At a total concentration of 1 g/100 ml. the apparent proportions were about equal. At a higher concentration the faster component appeared only slightly increased. But at low concentrations the apparent proportions were anomalous, showing too little of the faster component. The same patterns were produced with mixtures of oxyhaemoglobin from

different rabbits with oxyhaemoglobin from the same human adult. The descending patterns of the dilute solutions (Fig. 8) indicate that the faster peak is abnormally large, however, the spreading of the boundaries and the poor separation did not permit a quantitative analysis.

These results might be explained by an interaction (Longworth & MacInnes, 1942) between the two oxyhaemoglobins resulting in a smaller average migration velocity of the human oxyhaemoglobin in the presence of rabbit oxyhaemoglobin. On the ascending side, therefore, an increase in migration velocity of the faster component (human oxyhaemoglobin) at the boundary of the slower component necessitates a diminished concentration of the faster component above this boundary, as has been observed in the experiments. On the descending side the effect is reversed, in that the concentration of the slower component is reduced in the layer above the boundary of the faster component. This argument is formally equivalent to that used by Longworth & MacInnes, based on the assumption of a complex formation. Further support for this explanation is the lack of variation in the difference between the migration velocities with the HbO₂ concentration, at constant current, in the ascending limb (Table 4). This difference should become smaller with increasing concentration if there is no interaction.

The values for the difference in mobility of the boundaries in Table 2 and those in Table 4 disagree by more than can be accounted for by experimental error of the differential method. The small variation with changing oxyhaemoglobin concentration in the

differential method suggests little interference by boundary anomalies. In the experiments with oxyhaemoglobin mixtures, no change in concentration of the faster component across the boundary of the slower component appears to occur on the ascending side at medium oxyhaemoglobin concentration, the proportions of the refractive areas being the same as those of the components in the mixture. In an experiment set up as in Fig 1*a* at similar oxyhaemoglobin concentrations, a layer of human oxyhaemoglobin solution was put above the layer of the mixture, thus merely shifting the boundary of the faster component upwards by a distance equal to the length of one compartment, consequently on migration the change in the distance between the boundaries should have been identical with that obtained in the experiment with the mixture. This was not the case. The reasons for this discrepancy may be sought in the uncertainty of locating the boundaries in the experiments with the mixtures of oxyhaemoglobins. The boundaries are evidently non-symmetrical, and in more concentrated solutions indications of streaming can be observed at the slower peak (Fig 8, no 11) as the separation of the peaks becomes more complete. At the earlier stages of the run the partition line between the peaks cannot be drawn with the accuracy required, and at the later stages when this uncertainty diminishes it is the one-sided tailing off of the peaks which interferes. The tendency then is to exclude parts of the outer tail regions of the boundaries when locating the peaks, and accordingly the measured distance between them is too small. Although it appears that this error does not account for the whole of the discrepancy between the values in Tables 2 and 4, the remaining difference is too small to form a basis for any conclusions.

DISCUSSION

The boundary anomalies are at a minimum near the isoelectric point (Longsworth, Cannan & MacInnes, 1940) and they are probably negligible in an experiment with oxyhaemoglobins at pH 7.1, such as no 3 in Table 2. They are presumably not of great importance in the experiments made with oxyhaemoglobins at pH 8, since similar differences between the mobilities were obtained at concentrations of 0.66 and 0.05 g/100 ml. The close agreement in these experiments between the values for d obtained from the ascending and descending sides, cannot be taken as indicating that the boundary anomalies could be neglected, since these may have been effective in the same direction in both limbs of the U-tube (Svensson, 1946). However consistent the experimental values may be for the minimum differences in mobility under varying conditions, for theoretical reasons it is not possible to obtain accurate values by the differential method, although the approximation may be good if the relative difference in mobility is not small, and

if no interaction occurs between the components. The differential method might well be restricted for the present to establishing the identity of colloids, and to semi-quantitative statements on differences in mobility.

While the decision as to which of the components is the faster can be made with certainty from the observations by the differential method, such a decision may be misleading if the mobilities obtained in separate experiments are compared. This applies particularly when the mobilities are low, since slight deviations in the technique of reproducing the conditions in the electrode vessels may escape observation. In certain cases, e.g. in the comparison of serum and urine albumins, greater sensitivity could be obtained by the differential method than would be expected in absolute measurements of mobility.

The experiments described by Longworth & MacInnes (1940*b*), who found that the mobilities of the urine albumins from nephrosis patients, in lithium-veronal buffer of pH 7.8, were consistently lower than those of the serum albumin, are not comparable with the present experiments on account of the different buffers used.

The anomalous proportions of refractive areas in mixtures of human and rabbit oxyhaemoglobins appear to indicate an interaction between these two components. Anomalous patterns with dilute solutions were found in four experiments, in which the components had been in contact for different lengths of time (1 hr, 12 hr, 3 days, 6 days), and therefore an explanation cannot be sought in a slow reaction between the components. Haemoglobin molecules have been shown to split into halves in dilute solution (MacCarthy & Popják, 1947; Gutfreund, 1948). If such a reaction occurred with both human and rabbit oxyhaemoglobin under the conditions of the present experiments, the possibility would have to be considered of the formation of mixed molecules of human and rabbit oxyhaemoglobin on recombination of the fragments. A mixed molecule would presumably migrate with a mobility intermediate between those of the components and three peaks should appear in the pattern, such as has been observed by Tiselius & Horsfall (1939) in experiments with haemocyanins, or if the mobilities were little different the boundary might appear as a single broad peak.

No useful information concerning interaction between the two oxyhaemoglobins was gained by electrolysis of the mixture in phosphate buffer of pH 8 at the higher ionic strength of 0.2. The two components migrated as a single peak (Fig 8, nos 13–15). After dialysis of the same mixture against phosphate buffer of pH 8 and ionic strength 0.05, the patterns of Fig 8, nos 3 and 4 were obtained, showing two components as in the other experiments at this ionic strength.

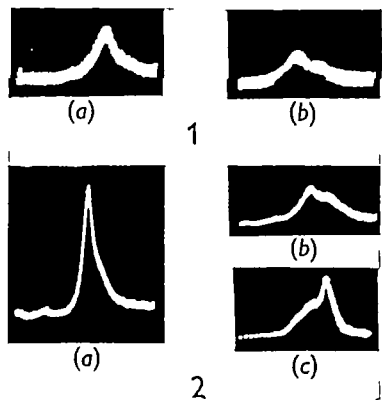


Fig 4.

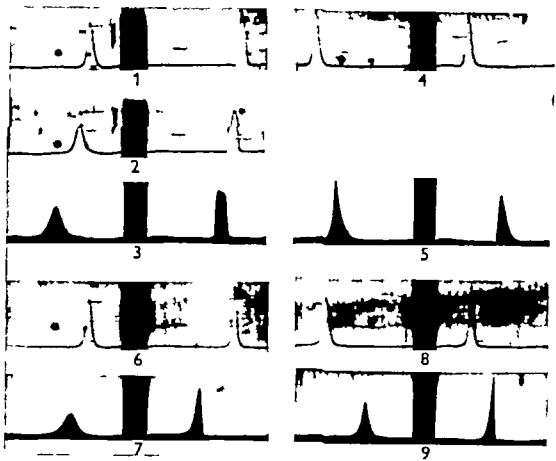


Fig 6

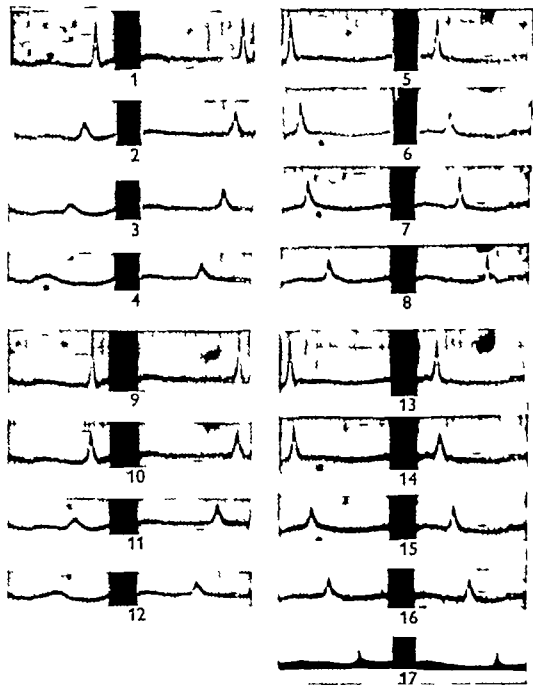


Fig 7

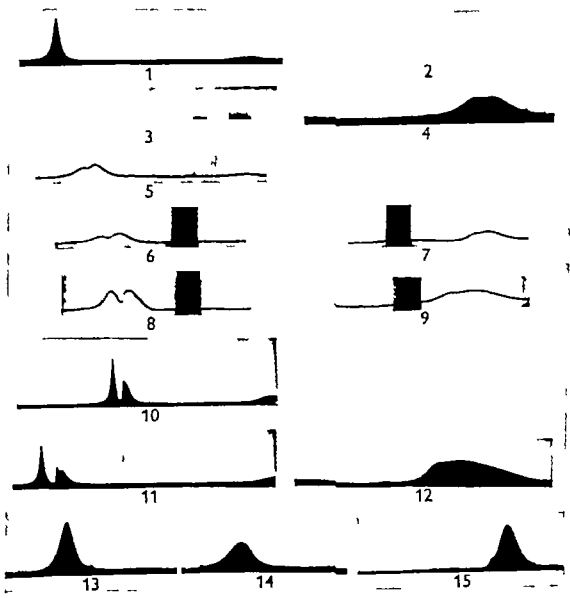


Fig 8

SUMMARY

1 An electrophoretic technique is described in which the migration of two colloid boundaries, formed at a distance from each other, can be observed simultaneously. Under limiting conditions the change in the distance between the two boundaries is a measure of the difference in mobility of the two colloids. The differential method described is considered to be supplementary to and, in particular cases, more sensitive than that of comparing the mobilities in separate runs. The main field of application offers itself in the establishment of identity of colloids.

2 A modified formula for the calculation of the position of the centroidal ordinate is given.

3 The method was applied to the study of serum and urine albumins, and of human and rabbit oxyhaemoglobins. Serum and urine albumins from three cases of nephritis showed a minimum difference in mobility at pH 8 of about 0.03×10^{-5} in two cases,

and of 0.42×10^{-5} in the third case. The urine albumin had the greater mobility. The minimum difference in the mobilities of human and rabbit oxyhaemoglobins was 0.4×10^{-6} at pH 8 and 0.33×10^{-6} at pH 7.1. The human oxyhaemoglobin had the higher anodic mobility. Oxyhaemoglobins from two normal adults showed no detectable difference in mobility.

4 Mixtures of human and rabbit oxyhaemoglobins of known composition gave patterns with two main peaks at pH 8 and ionic strength 0.05 in which the proportions of the refractive areas were abnormal. At pH 8 and ionic strength 0.2 the two components did not separate.

I wish to thank Prof J. R. Marrack for encouragement and for criticism. I am greatly indebted to Prof A. Tiselius for his interest and for hospitality in his laboratory, where I had the privilege of conducting part of this work, and to Docent H. Svensson, with whom I had the pleasure of many discussions of the problems involved. I should also like to thank Dr E. M. Jope for his help in connexion with the work on haemoglobin.

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EXPLANATION OF PLATE 3

Fig 4 Electrophoretic patterns of serum and urine albumins in nephritis. Left, serum albumin, right, urine albumin. 1(a) and (b) ascending patterns from case 2 of Table 1. 2(a) and (c) ascending (b) descending patterns from case 3 of Table 1. Ascending patterns after 7-9 hr, direction of migration towards the right. 2(b) after 3 hr, direction of migration towards the left. phosphate, pH 7.7, μ , 0.1, 9.5 V/cm.

Fig 6 Electrophoretic patterns obtained by the differential method in Exp 3 of Table 2. Run I, set up as in Fig 1(d) nos 1-3 ascending patterns, human HbO₂ in upper layer, after 60, 177 and 323 min migration. Nos 4 and 5 descending patterns, rabbit HbO₂ in upper layer, after 63 and 327 min. Run II, set up as in Fig 1(e) nos 6 and 7 ascending patterns, rabbit HbO₂ in upper layer, after 61 and 397 min, nos 8 and 9 descending patterns, human HbO₂ in upper layer, after 64 and 394 min. The direction of migration for the ascending patterns is towards the left, for the descending towards the right. In each run all four boundaries were of equal height and identical shape at the beginning of the experiment. Phosphate, pH 7.1, μ , 0.08, 8.7 V/cm, HbO₂ concentration in upper layer, 0.5 g/100 ml.

Fig 7 Electrophoretic patterns obtained by the differential method in Exp 2 of Table 2. I nos 1-4 ascending patterns, human HbO₂ in upper layer, after 0, 33, 66 and 132 min migration. Nos 5-8 descending patterns, rabbit HbO₂ in upper layer, after 0, 36, 69 and 135 min. II nos 9-12 ascending patterns, rabbit HbO₂ in upper layer, after 0, 9, 58 and 111 min. Nos 13-17 descending patterns,

human HbO₂ in upper layer, after 0, 12, 60, 113 and 185 min. The direction of migration for the ascending patterns is towards the left, for the descending towards the right. Phosphate, pH 8, μ , 0.05, 10 V/cm, HbO₂ concentration in upper layer, 0.05 g/100 ml.

Fig 8 Electrophoretic patterns of oxyhaemoglobins. All experiments, with the exception of nos 13-15, in phosphate, pH 8, μ , 0.05, 14 V/cm. Nos 1 and 2 ascending and descending patterns of rabbit HbO₂, concentration = 1.35 g/100 ml, after 430 and 435 min. Nos 3-15 patterns of mixtures 1:1 of human and rabbit HbO₂.

Left, ascending limb, right, descending limb. Nos 3 and 4 = Exp 1 of Table 4, after 320 and 322 min, no 5 = Exp 2, after 299 min, nos 6 and 7 = Exp 3, after 321 and 209 min, nos 8 and 9 = Exp 6, after 382 and 385 min, nos 10-12 = Exp 7, after 243, 360 and 364 min, nos 13 and 14 ascending patterns, no 15 descending pattern of a mixture 1:1 of human and rabbit HbO₂ in phosphate pH 8, μ = 0.2, nos 13 and 15 after 443 min at 7 V/cm, then 160 min at 6 V/cm, no 14 after 443 min at 7 V/cm, then 173 min at 6 V/cm, then 815 min at 4 V/cm. Total distance migrated about 9 cm.

Chromatographic Studies of Nucleic Acids

1 A TECHNIQUE FOR THE IDENTIFICATION AND ESTIMATION OF PURINE AND PYRIMIDINE BASES, NUCLEOSIDES AND RELATED SUBSTANCES

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(Received 31 March 1949)

Until recently the isolation and identification of purine and pyrimidine bases, ribosides and nucleotides in mixtures such as nucleic acid hydrolysates were only possible by purely chemical methods of separation, necessitating the use of very large quantities (often 100 g or more) of starting materials (Levene & Bass, 1931). Such methods are tedious, very limited in application and far from quantitative. However, the development of partition chromatography on paper (Consden, Gordon & Martin, 1944) has afforded the possibility of the separation of mixtures of purine and pyrimidine derivatives in minute amounts. The successful application of this method depends upon the location on the paper chromatogram of the areas occupied by the various substances. Once located, these areas may be cut out, extracted in a suitable solvent, and the substances estimated by ultraviolet spectrophotometry, making use of their characteristic absorption bands in the region of 260 m μ . Two methods of detecting purine and pyrimidine derivatives have been described recently. Hotchkiss (1948) cut the paper into transverse strips, 5-30 mm wide, extracted each of these in water and, using a Beckman photoelectric spectrophotometer, determined for each extract the absorption in the ultraviolet region near 260 m μ . This method is time consuming, does not allow of a very high resolution of adjacent spots, and for practical reasons is obviously inapplicable to two dimensional chromatograms. In a second method, described by Vischer & Chargaff (1948a), the purines and the pyrimidines are converted on the chromatogram to mercury complexes. Excess

mercury is removed and the paper is treated to convert the mercury in the complex into mercuric sulphide, which being black indicates the position of the spots. While this technique allows the detection of as little as 5 μ g of the free bases in individual spots, it has serious limitations. The method is not rapid, involving prolonged treatment of the paper in the wet state with several reagents and as described is limited to the bases. Furthermore, during the conversion of the mercury complexes into mercuric sulphide, the bases are lost so that for quantitative analysis parallel untreated strips of the chromatogram have to be used. It is thus impossible to use this technique for quantitative two dimensional chromatograms.

To avoid these difficulties we have developed a simple and rapid method which will detect in a chromatogram all substances having a strong absorption in the ultraviolet near 260 m μ . Such substances, of course, include all purine and pyrimidine derivatives, as well as many other compounds of biological interest. The basis of this method, which has been reported briefly elsewhere (Markham & Smith, 1949) is a simple photographic method for the detection of absorbing substances.

METHODS

(a) Location of the spots

As all purine and pyrimidine derivatives have an intense ultraviolet absorption in the neighbourhood of 260 m μ it is natural to look for methods suitable for exploiting this characteristic. Filter paper happens to be reasonably

transparent in this region, so it is possible to locate the spots of absorbing substances merely by making a contact print by filtered ultraviolet light on to sensitized paper through the dried paper chromatogram

The source of ultraviolet light used in these experiments was a Hanovia 'Homesun' lamp, a 450 W, 200 V, mercury arc lamp. This lamp has a high emission at $253.7\text{ m}\mu$, a wavelength which, though not ideal, is satisfactory for most purposes. In order to make the method as sensitive as possible it is desirable to use filters in order to obtain light of this wavelength as nearly monochromatic as possible, particularly as photographic materials are very sensitive to the longer wavelength ultraviolet light emitted by this lamp. Several filters are available for this purpose, but for convenience a liquid filter containing CoSO_4 and NiSO_4 ,

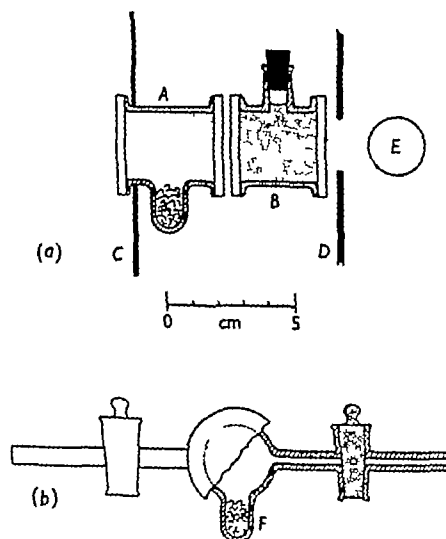


Fig 1 a, Longitudinal section of the chlorine filter (A) and liquid filter (B) showing their design. C and D are diaphragms and E is the mercury vapour lamp. b, Partial transverse section of chlorine filter showing taps used for filling and, F, calcium chloride for keeping the gas dry

followed by a gaseous chlorine filter, was chosen (Bowen, 1946). The filters are of glass with fused quartz windows cemented on. For the liquid filter a resinous cement is used, but for the chlorine filter vaseline is used, a few dabs of Piccin wax round the outside holding the assembly firm. The liquid filter, which contains an aqueous solution of $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$, 350 g and $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 100 g/l., is 30 mm thick and the gas filter containing chlorine at atmospheric pressure dried over CaCl_2 is 35 mm thick. The cells have a free aperture of 22 mm and their design is shown in Fig 1. It seems to be important to have the liquid filter nearer the lamp, as otherwise the chlorine gas will need renewing frequently, whereas if they are arranged as recommended the gas persists indefinitely. A useful check on the efficiency of the filters is given by observing the spectrum of the light transmitted with a Beck ultraviolet spectroscope. The only lines visible should be at 253.7 and $265\text{ m}\mu$, the latter being much less intense. If such an instrument is not available, it is possible to test the light source by printing out a few test spots of adenine on Whatman no 1 paper. It should be

possible to detect $1\text{ }\mu\text{g}$ of adenine in a $10\text{ }\mu\text{l}$. spot quite easily, whilst $5\text{ }\mu\text{g}$ in the same volume should prove almost completely opaque. It is, in fact, possible to detect quantities of materials which are much too small to estimate accurately by means of a spectrophotometer.

The photographic material used is Ilford Reflex Document Paper, no 50 (similar material is also manufactured by other firms). This material is comparatively cheap, and has the advantage of being extremely contrasting, and it may, if necessary, be handled in subdued artificial light. It is not affected by the yellow light transmitted by the filter system. (Other papers, though faster, have not proved so sensitive to small amounts of absorbing material.) We have not found that the developers used modify the results greatly and either the maker's formula I D 36 or a process developer (Kodak D153) seems quite satisfactory.

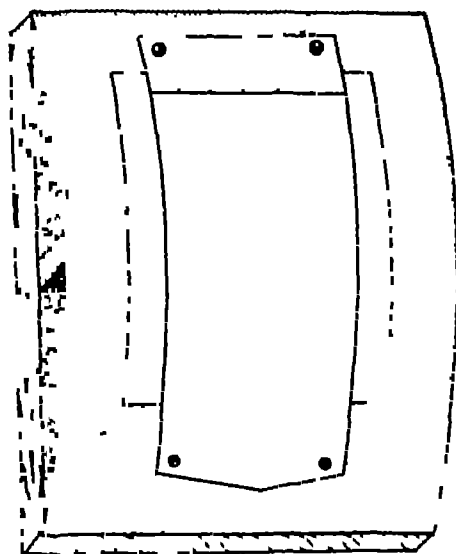


Fig 2 Printing the chromatogram. The photographic paper is held flat by the filter paper chromatogram which is pinned over it on the convex surface of the plywood.

Exposure. Under our conditions a suitable exposure is 15 min at a distance of 1.2 m (which covers an area of about $15 \times 40\text{ cm}$). For larger areas such as are needed for two dimensional work, the light source has to be farther away, and the exposure may be estimated approximately by applying the inverse square law. The exposures are not very critical.

For making the prints it would be desirable to have a printing frame of sufficient size with a fused quartz window, but for all practical purposes we find that pinning the chromatogram paper over the photographic paper on a plano convex surface (a piece of plywood fastened to a board with a small strip of wood under the centre to bow the former out a little (Fig 2)) is entirely satisfactory.

After development the paper is fixed and washed in the usual way. If it is necessary to dry the paper rapidly it should be bathed after washing in ethanol or acetone and then dabbed with a clean cloth. Dimensional changes in the print appear to be negligible, but if they should be a source of trouble similar emulsions may be obtained coated on water proof paper, cellulose acetate or glass.

The positions of absorbing substances may be seen on the developed papers as light areas (Fig 3) and these prints serve as a permanent record of each experiment

For many purposes the photographic image alone will suffice, but if the spots need to be extracted, their positions may be found by shining a light through the photographic paper and drawing their outlines on the filter paper, which is registered by means of pencil lines or other reference marks made on the latter and which show up on the print. As a general routine we write all the necessary data in pencil on

investigated (excluding certain methylated derivatives) were less than 0.5, the lower edge of the paper was cut to a point and the solvent front was run well beyond the end of the paper and a substance of known R_F value, usually adenine, was used as a marker. Two dimensional chromatograms were run on standard sheets of filter paper in large glass sided boxes. Solutions were placed on the paper in small areas 6 cm from the upper edge, either with small glass capillary tubes, or, for quantitative work, with a Burroughs Wellcome Agla syringe. Volumes of 10–20 μ l of solutions containing less than 50 μ g of individual substances were used.



Fig 3 Print of chromatogram run for 9 hr in *n* butanol formic acid to illustrate points in text. Mixtures were put on at spots 1–5 and each column reads from the top downwards (1) Uric acid, 6.8 dihydroxypurine and hypoxanthine, and (2) guanine and adenine, both showing effect of OH groups, (3) adenosine and adenine, and (4) cytidine and cytosine, showing effect of ribose, (5) xanthine, 3 methylxanthine, 3.7 dimethylxanthine (theobromine) and 1.3.7 trimethylxanthine (caffeine) showing effect of methylation. (Note To resolve all these substances when present together several solvents would have to be used and the chromatograms run for longer times.)

the filter paper so that it is recorded on the print as well. The areas of filter paper corresponding with the spots on the photographic print are cut out and extracted in 5 ml of water or 0.1N HCl for estimation in a Beckman photo electric spectrophotometer. Areas identical in size are cut from adjacent strips for use as blanks (see Hotchkiss, 1948).

(b) Chromatographic technique

All chromatograms were run on Whatman no. 1 paper. For single dimensional work sheets 13 × 33 cm. were run in glass battery jars. As the R_F values of most of the substances

(c) Choice of solvent

Naturally in this work solvents such as phenol, quinoline, collidine, etc., which absorb heavily in the ultraviolet and are difficult to remove from the paper must be avoided if possible. Several aliphatic alcohols and ketones were tried, but *n* butanol saturated with water (14% water in butanol (v/v), Hotchkiss, 1948) proved the most useful basic solvent for the separation of the bases and the nucleosides. For a satisfactory differentiation between hydroxy and amino purines and pyrimidines (Fig 3), either ammonia was added to the vapour phase or the solvent was acidified. The systems used most frequently were (1) a mixture containing 90% *n* butanol saturated with water and 10% glacial formic acid (v/v), (2) *n*-butanol saturated with water in the presence of an atmosphere containing NH_3 , (3) *n* butanol saturated with water. For two-dimensional chromatograms a combination of (1) and (2) will separate all the substances likely to be encountered in nucleic acid hydrolysates (including xanthine and hypoxanthine, traces of which are usually present).

(d) Materials

Cytidine and uridine were obtained from Dr J. Davoll and yeast adenylic acid from Dr T. Mann. The methylated xanthines and 6.8 dihydroxypurine were from Prof. D. Keilin, F.R.S. Cytosine was obtained by hydrolysis of cytidine at 145° in 6N HCl. Though hydrolysis was practically complete no trace of uracil was found, although we have confirmed the observation of Vischer & Chargaff (1948b) that during the acid hydrolysis of nucleic acids some uracil is formed from cytosine. Thymine was obtained from thymus nucleic acid by hydrolysis. The other substances were commercial products.

RESULTS

Table 1 gives the positions on the chromatograms of purines and pyrimidines and some of their derivatives in various solvents. This table is only intended as a guide to the relative positions of these substances, as we find a certain amount of variation from time to time. For instance, particularly in butanol ammonia systems, the pyrimidines, uracil and cytosine (and their corresponding ribosides) are very sensitive to changes in the composition of the vapour phase and they may change their relative positions. In such chromatograms it is necessary to use adenine and uracil as markers. In the butanol formic acid system, adenine and hypoxanthine may undergo a similar displacement. When running two-dimensional chromatograms it is essential to use

Table 1 R_f values of purines, pyrimidines and ribosides in various solvents

Solvent system*	n Butanol 86% water 14%	n Butanol 86% water 14% +NH ₃ †	n-Butanol 77% water 13% formic acid 10%	n Butanol 50% ethanol 15% water 35%	Amyl alcohol saturated with water	Amyl alcohol saturated with water + NH ₃ †	Amyl alcohol saturated with water 90% formic acid 10%
Purines							
Hypoxanthine	0.26	0.12	0.30	—	0.15	0.02	0.19
Xanthine	0.18	0.05	0.24	—	0.10	0	0.16
6,8 Dihydroxypurine	0.18	0.04	0.24	—	0.07	0	0.11
Uric acid	0.01	0	0.14	—	0	0	0.06
3 Methylxanthine	0.29	0.13	0.32	—	0.19	—	—
1,3 Dimethylxanthine (theophyllin)	0.52	0.22	0.64	—	0.54	0.12	0.55
3,7-Dimethylxanthine (theobromine)	0.42	0.27	0.47	—	0.28	0.15	0.36
3,8 Dimethylxanthine	0.42	0.18	0.48	—	0.34	—	—
1,3,7 Trimethylxanthine (caffeine)	0.63	0.65	0.71	—	0.56	0.50	0.67
Adenine	0.38	0.28	0.33	0.55	0.28	0.16	0.12
Guanine	0.15	0.11	0.13	0.37	0.05	—	0.04
Purine ribosides							
Adenosine	0.20	0.22	0.12	0.50	0.11	0.09	0.04
Guanosine	0.15	0.03	0.17	0.40	0.02	0	0.04
Pyrimidines							
Cytosine	0.22	0.24	0.26	0.53	0.09	0.09	0.07
Uracil	0.31	0.19	0.39	0.46	0.22	0.08	0.23
Thymine	0.52	0.35	0.66	—	0.40	—	—
Pyrimidine ribosides							
Cytidine	0.12	0.11	0.18	0.42	0.02	0.03	0.03
Uridine	0.17	0.08	0.25	0.49	0.05	0.03	0.07

* Composition of solvents is given in terms of volume percentages

† 5% by vol. of NH₃ solution (sp gr 0.880) was added to the solvent mixture in the bottom of the container

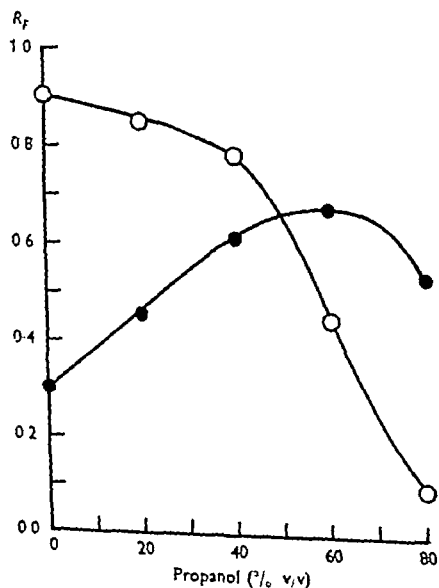


Fig 4 The variation of R_f values of adenylic acid (open circles) and adenine (solid spots) in solvents of varying water content

separate markers in each dimension. A print is then taken of the markers after running in each solvent. The markers are, of course, placed on the other end of the top edge of the filter paper sheet and thus do not interfere.

Solvents which contain little water do not bring about much movement of the phosphorylated derivatives (nucleotides, dinucleotides, etc.) owing to the much greater relative solubility of these substances in water, and consequently they are to be found at the position of the original spot in butanol-water chromatograms. Some separation of these substances may be obtained by the use of solvents based on ternary mixtures such as butanol-ethanol-water and on completely miscible solvents such as propanol-water (Fig 4).

DISCUSSION

From Table 1 it appears that certain generalizations as to the movement of substances containing certain groupings may be made, and we enumerate them as follows:

(1) Pyrimidines move faster than the corresponding purines. For example, uracil runs faster than xanthine and cytosine faster than guanine.

(2) In the presence of ammonia the amino derivatives move much faster than the corresponding hydroxy compounds, but on changing to an acidic solvent this difference is greatly decreased and the relative positions may even be reversed. This is particularly evident in the case of the pyrimidines cytosine and uracil (and their ribosides) in which the reversal takes place at neutrality.

(3) Increase in the number of hydroxyl groups possessed by a substance decreases its rate of movement in all the solvents. This is illustrated by a comparison of the R_f values of hypoxanthine, 6,8-dihydroxypurine and uric acid, and those of adenine and guanine, adenosine and guanosine.

(4) Ribosides move more slowly than the free bases but maintain the same relative positions.

(5) Increasing methylation tends to increase the movement of the substances in all the solvents.

Comparing these results with those of other workers some points of interest may be noted. We find, contrary to Hotchkiss (1948), that, when present in amounts small enough to be completely in solution in the aqueous phase, guanine moves at an appreciable rate in butanol mixtures, and probably corresponds with the 'epiguanine' to which

he refers. This is probably due to the fact that his method has too poor a resolution to give a satisfactory picture of the chromatogram as a whole.

Except in acidic solvents, uracil always runs in our chromatograms more slowly than adenine, contrary to the observations of Vischer & Chargaff (1948a). These and other minor discrepancies would appear to be due, in part at least, to the different filter paper used by these authors.

SUMMARY

1. A micromethod for the detection and estimation of purines, pyrimidines and related substances is described. It is based upon the detection of spots of these compounds on paper chromatograms by means of a simple contact printing technique using photographic paper and filtered ultraviolet light, and the sensitivity is such that a few micrograms may be detected.

2. The R_f values of a number of compounds in several solvents are tabulated.

3. Certain generalizations are made correlating chemical structure with the chromatographic behaviour of these substances.

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Studies in Vitamin A

10 VITAMIN A₁ AND RETINENE₁ IN RELATION TO PHOTOPIC VISION

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(Received 11 February 1949)

Scotopic (or low intensity) vision is related to the activity of rods, and photopic (or daylight) vision operates by means of cone receptors. When light falls only on rods there is no sensation of colour, and the spectral distribution of sensitivity in scotopic vision can be interpreted in terms of one receptor substance, rhodopsin. For photopic vision, however, the phenomena of colour sensation cannot be explained on the basis of a single absorbing entity with a spectrum corresponding to the photopic sensitivity curve. Nevertheless, the full cone response curve obtained by electrophysiological

methods is very similar to the normal photopic sensitivity curve.

One interpretation of the Young-Helmholtz theory is that it implies the existence of three selectively absorbing substances sensitive to different spectral ranges. Many workers have attempted to measure the responses of different receptors to different wavelengths. The usual procedure was first to expose the eye to filtered (or better monochromatic) light in the hope that the receptor affected would be put out of action by photochemical exhaustion. The response of another receptor to

wavelengths of monochromatic light within its own range of sensitivity could then be determined. Prolonged exposure of the eye to one wavelength, however, always produced a reduced response to all wavelengths.

Since 1936, Granit has studied the problem by electrophysiological methods (for review see Granit, 1947). The complicating rod phenomena were eliminated by pretreatment of the eye with light sufficiently intense to bleach all the rhodopsin. After removing the lens and cornea, and applying micro-electrodes directly to the retina, Granit was able to pick up responses from individual nerve fibres when monochromatic light fell on the retina, and in that way made fundamental advances in the combined problems of colour sensation and photopic vision. His scotopic action spectra for frog, toad, cat, rabbit, pigeon, guinea pig, fresh-water eel, etc. were very uniform and enabled him to compute the absorption spectrum of rhodopsin. With light-adapted eyes striking results were obtained. Sensitivity curves widely different from the normal photopic sensitivity curve were often recorded, and as a result of many different series of experiments Granit advanced his dominator modulator theory.

A dominator is a sensory mechanism, whether scotopic or photopic, characterized by a broad sensitivity curve and making available for vision a large range of wavelengths. The differences between the photopic and scotopic dominators account for the Purkinje shift, and the scotopic dominators correspond with rhodopsin or porphyropsin, depending upon whether the eye under study makes use of vitamin A₁ or vitamin A₂. The fact that the photopic dominator response curves are always displaced by some 60–70 mμ in the direction of longer wavelengths compared with the corresponding scotopic curves whether the eyes contain vitamin A₁ or A₂ or both, suggests that the receptor substances may all be related to the vitamins A. The dominator curves are concerned with the sensation of brightness and are too broad to allow them an important role in colour discrimination.

A modulator, on the other hand, is characterized by a much narrower response curve. The substantial body of data accumulated by Granit shows that the modulator action spectra fall into groups situated in three spectral regions: 440–470, 520–540 and 580–600 mμ. There is an added complication in the existence of a light-sensitive modulator near 500 mμ. The most light-resistant modulator appears to be that near 600 mμ. The narrow action spectra make the modulators peculiarly suitable for colour sensitivity. There is independent evidence that colour mechanisms are not responsible for brightness discrimination (Wright & Granit, 1938).

The outstanding implication of Granit's work, from the present point of view, is that his action spectra provide serviceable 'labels' for hypothetical light absorbing substances concerned in vision, provided that the relationship between absorption spectra and action spectra is borne in mind (see Ball, Collins, Morton & Stubbs, 1948; Dartnall & Goodeve, 1937; Hecht, 1937; Wald, 1938).

Von Studnitz (1932) bleached isolated retinas and measured the changes in light absorption at different wavelengths, but the evidence he produced for a photosensitive cone pigment is technically questionable. He later (von Studnitz, 1937) extracted from frog retinas an ether soluble material with λ_{\max} 560 mμ, but when his data are re-plotted on a scale suitable for comparison with photopic sensitivity it seems that the absorption curve was broader than the 'dominator' curve. For the tortoise (*Testudo graeca*) he recorded λ_{\max} for the extract at 560 mμ, although the photopic sensitivity maximum is at 600–610 mμ. Using the snake *Tropidonotus* he obtained extracts with absorption maxima at 468, 560 and 650 mμ. Chase (1938) extracted a water soluble cone substance (absorption maximum 530 mμ), and Wald (1938) deduced from differential photo-decomposition of chicken retinas an absorption maximum at 575 mμ, which he attributed to a cone pigment, iodopsin. Hosoya, Okita & Akune (1938), using the rod free retinas of tortoises, claimed to have extracted by means of 2% sodium cholate three water soluble pigments the absorption spectra of which showed maxima at 460, 570 and 670 mμ, respectively, but the data were not very satisfactory. Hanström (1940) extracted the macular region of monkey retinas and found maxima at 460 and 590 mμ due to ether soluble substances.

Blass (1948a, b) has confirmed the existence of Wald's iodopsin in chicken retinas and has been able to extract it, although not in a very 'pure' state. The iodopsin was more labile than rhodopsin, and bleaching by light produced retinene in 'large' amount, but there was no detectable change in pH or inorganic phosphate concentration.

That a deficiency of vitamin A impairs scotopic vision is by now familiar, but the relationship of vitamin A to photopic vision is not so clear cut. Inasmuch as there is no real hint of any other key substance in vision, the next step is to find out whether vitamin A is capable of being the only key substance.

Vitamin A and retinene give rise to deep blue solutions with antimony trichloride in chloroform, and the sharpness of the absorption bands, coupled with the transient nature of the blue materials, suggests that under suitable environmental conditions vitamin A or retinene might give rise to 'ionized' or 'halochromic' molecules (see Meunier & Vinet, 1947) resembling the modulators of Granit. When vitamin A or retinene is dissolved in concentrated sulphuric acid or syrupy phosphoric acid at temperatures near 0°, coloured solutions are produced which exhibit well-defined selective absorption with maxima corresponding closely with Granit's maxima. These results are recorded and discussed below.

EXPERIMENTAL

The materials used were crystalline samples of vitamin A alcohol and retinene, the ionizing media being H₂SO₄ (sp gr 1.84) and H₃PO₄ (sp gr 1.75). The Beckman spectro-photometer was used for the quantitative measurement of absorption spectra, the Hilger Nutting visual instrument for some qualitative determinations.

RESULTS

Vitamin A and sulphuric acid

A few minute crystals of vitamin A were added to about 20 ml of concentrated sulphuric acid and the mixture was well shaken. The vitamin dissolved easily, and a transient purple colour was immediately formed (λ_{\max} 620 $m\mu$). The blue component of the colour very quickly faded, and the solution became bright red with absorption maxima at 465, 520 and 580 $m\mu$. By means of the Hilger Nutting instrument the diminution in intensity and disappearance of the 620 $m\mu$ band with concomitant appearance and increasing intensity of the 580 $m\mu$ band could be observed, showing clearly an interrelationship between the substances responsible for these absorption maxima. The 520 $m\mu$ band was also unstable and disappeared fairly rapidly at room temperature even in the dark. If the sulphuric acid solution was kept in the solid state at -78° in the dark, all three bands persisted for several days. Under such conditions, i.e. dissolution of vitamin A in very cold sulphuric acid, an absorption band was found at 560 $m\mu$ instead of 580 $m\mu$.

When, immediately after mixing, the sulphuric acid solution of vitamin A was agitated with light petroleum, cyclohexane or ether, coloured materials did not pass into the organic solvent and ultraviolet absorption measurements on the extracts showed only the presence of unchanged vitamin A. When chloroform was used as the extracting solvent a cloudy solution was obtained which developed a pink colour on standing. The absorption spectrum of this chloroform extract (Fig 1) showed bands at 460 and 555 $m\mu$, due to separate chemical entities, since the absorption bands disappeared at different rates under the influence of heat or light.

A dilute dispersion of concentrated sulphuric acid in chloroform was prepared by shaking a mixture of chloroform and sulphuric acid vigorously for 10 min and allowing to settle. The chloroform solution was slightly cloudy and an intense bluish red colour was immediately obtained on addition of vitamin A. The absorption spectrum of this coloured solution (Fig 2) was complicated and exhibited maxima at 470 and 570 $m\mu$. On standing for 15–25 min in light or the dark the spectrum was resolved into a series of absorption bands.

A solution of vitamin A in ethanol gave an intense blue colour with sulphuric acid (λ_{\max} 620 $m\mu$), which changed rapidly to a bright red (λ_{\max} 560 $m\mu$). This red colour also disappeared very quickly leaving a dirty brown solution which showed no selective absorption in the visible or ultraviolet.

Attempts were made to fractionate the coloured products of the sulphuric acid reaction, but without much success. Dilution with water at 0° , followed

by neutralization with sodium carbonate or barium carbonate produced colourless solutions exhibiting no selective absorption in the ultraviolet. The best

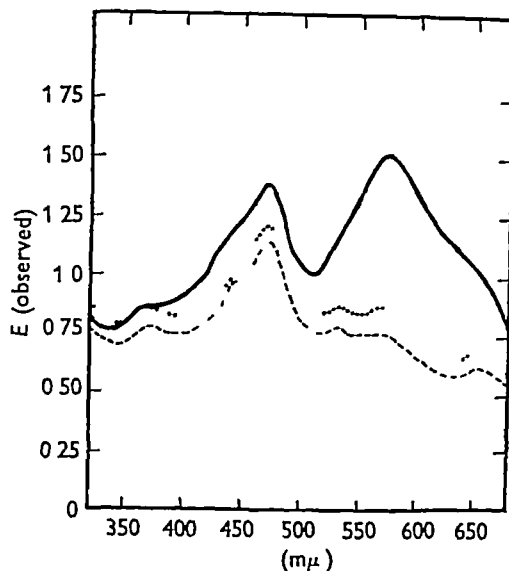


Fig 1 Absorption spectrum of chloroform extract of a solution of vitamin A in sulphuric acid —, measured immediately, ---, after 24 hr in the dark, . . . , after 24 hr in diffused daylight

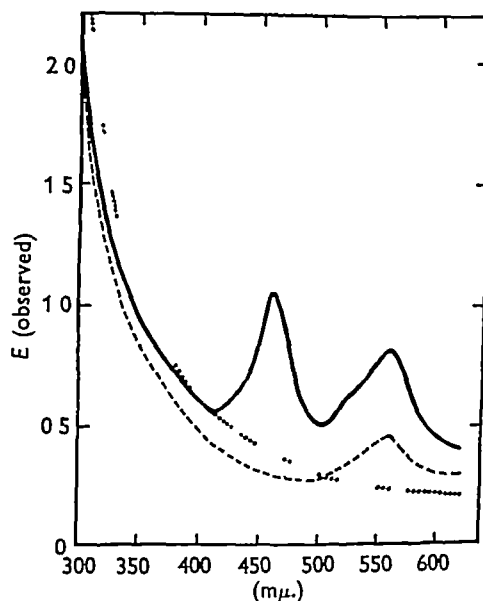


Fig 2 Absorption spectrum of vitamin A in a chloroform dispersion of sulphuric acid —, measured immediately, ---, after 15 min of diffused daylight, . . . , after 25 min in the dark

results were obtained by mixing solutions of vitamin A in ethanol with sulphuric acid at temperatures just high enough to keep the acid liquid

These mixtures were diluted with ice and water, treated with anhydrous sodium carbonate until effervescence ceased, and then extracted with chloroform. The chloroform solutions obtained in this manner were bluish green in colour, the absorption spectra showing maxima at 380 and 465 $m\mu$. The coloured products were stable at -80° in the dark, but faded rapidly on exposure to light or warming to room temperature.

Vitamin A and phosphoric acid

A sample of vitamin A ($E_{1\%}^{1\text{cm}}$ 980 at 326 $m\mu$ in cyclohexane) dissolved in a little ethanol, was stirred into phosphoric acid. A blue solution (λ_{max} 620 $m\mu$) was obtained at first, but rapidly became red with absorption maxima at 480, 540 and 600 $m\mu$ (Fig 3).

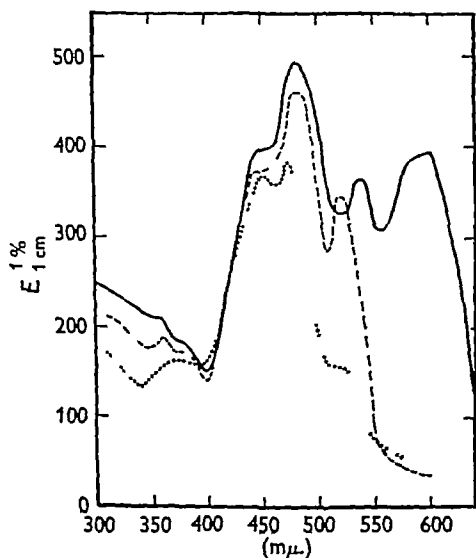


Fig 3 Absorption spectrum of vitamin A in phosphoric acid —, measured immediately, - - -, after 5 hr in the dark, . . . , after 5 hr in diffused daylight

The 600 $m\mu$ maximum disappeared quickly at room temperature in the dark, but the 480 $m\mu$ band persisted under these conditions. However, the 480 $m\mu$ band decreased in intensity on exposing the solution to light, and after 5 hr absorption bands of approximately equal intensity were found at 450 and 470 $m\mu$. After 5 hr in the dark, the 540 $m\mu$ maximum was displaced to 520 $m\mu$, while a similar sample exposed to the light showed only an inflexion at 520 $m\mu$.

Retinene and sulphuric acid

If retinene is dissolved in chloroform and the solution shaken with sulphuric acid, the acid layer becomes bright red in colour and the chloroform deep blue (λ_{max} 664 $m\mu$). Using light petroleum as the solvent for retinene, no colour develops in the

petroleum phase, although the acid layer again becomes red. Treatment of solid retinene with sulphuric acid gave a purplish red solution, the blue component of which disappeared rapidly, leaving a red colour. A complex absorption spectrum was observed with narrow absorption bands at 380, 440, 460, 520 and 560 $m\mu$ (Fig 4). These absorption maxima disappeared rapidly at room temperature, but persisted well at -80° . After dilution with ice and water, the acid was neutralized with anhydrous sodium carbonate and the solution shaken with chloroform. A colourless chloroform extract was obtained, the spectrum showing no selective absorption in the ultraviolet region. When this chloroform solution was treated with the antimony trichloride reagent a red coloration was obtained,

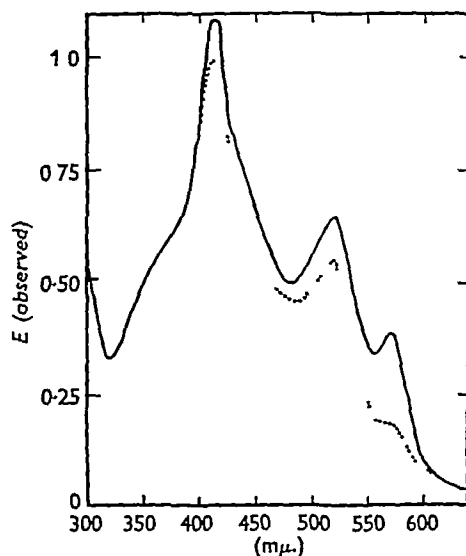


Fig 4 Absorption spectrum of retinene in sulphuric acid —, measured immediately, - - -, after 5 min. in diffused daylight

the absorption spectrum showing maxima at 495, 525 and 590 $m\mu$.

When a solution of retinene in ethanol was stirred into sulphuric acid, the temperature being just above the melting point of the acid, an intense blue colour was obtained (λ_{max} 664 $m\mu$). This rapidly changed to ruby red, absorption maxima being observed at 420, 520 and 570 $m\mu$. The solution was diluted, neutralized and extracted as described above, the chloroform extract showed no selective absorption in the visible and ultraviolet regions of the spectrum. With the antimony trichloride reagent a red solution was obtained, the absorption spectrum of which showed bands at 495, 525 and 590 $m\mu$ as above. In the original acid solution it was observed that exposure to light reduced the intensity of each

absorption band, the 570 $m\mu$ maximum diminishing more rapidly than the others. After 5 min in diffuse daylight the 570 $m\mu$ band had disappeared, whereas the other bands were still quite intense.

With sulphuric acid dispersed in chloroform retinene gave a blue solution (λ_{\max} 664 $m\mu$) which soon became red. The absorption spectrum then exhibited maxima at 390, 515 and 560 $m\mu$, all three bands being reduced in intensity by exposure to light or standing in the dark at room temperature.

Retinene and phosphoric acid

Solid retinene dissolved readily in syrupy phosphoric acid to produce a red solution, a transient blue colour being formed first. The coloured substances were markedly unstable unless the solution was kept at -80° , but increased stability was achieved by dissolving the retinene in a little ethanol

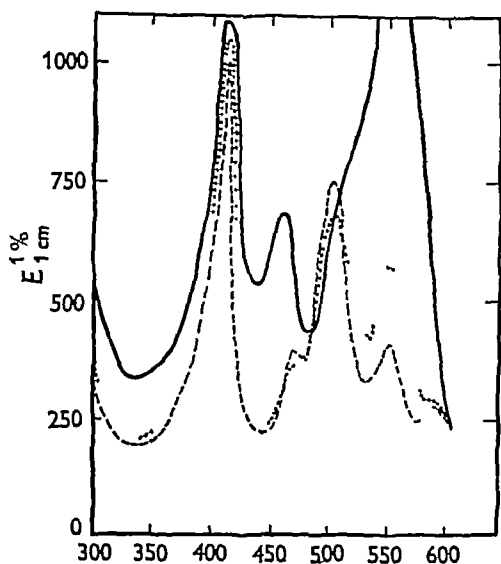


Fig 5 Absorption spectrum of retinene in phosphoric acid —, measured immediately, - - -, after 2 hr in the dark at 0° , . . . , after 2 hr in diffused daylight at room temperature

before adding the acid. The blue coloration was then recognized as due to a substance with λ_{\max} at 664 $m\mu$, and further bands were found at 410, 460 and 560 $m\mu$ in the absorption spectrum measured shortly after mixing (Fig 5). A pronounced inflexion was observed at 520 $m\mu$. After the solution had been left in the dark at 0° for 2 hr the absorption spectrum was remeasured. The 560 $m\mu$ maximum had decreased in intensity by more than half, the 460 $m\mu$ maximum had disappeared and the 520 $m\mu$ inflexion had become a sharp maximum at 500 $m\mu$. The 410 $m\mu$ maximum had remained unchanged during this time, and new absorption bands of low intensity were apparently responsible for the pro-

nounced inflexions at 470 and 585 $m\mu$. These changes are recorded in Fig 5, together with the results obtained when a similar solution was kept at room temperature in diffuse daylight for 2 hr.

A quantitative determination of the intensity of the 560 $m\mu$ maximum was rapidly carried out, the starting material being retinene of $E_{1\%}^{1\text{cm}}$ 1400 at 383 $m\mu$ in ethanol. Although the band was decreasing rapidly during the measurement, an $E_{1\%}^{1\text{cm}}$ value of 2480 was obtained at 560 $m\mu$. It dropped to 1884 in 3 min, and in 2 hr there was no maximum at that wavelength. Many similar measurements were made, but it was difficult to distinguish sharply between thermal and photochemical processes. It seems, however, probable that the substances responsible for the 410 and 500 $m\mu$ bands are stable to light even at room temperature, while the 560 and 460 $m\mu$ materials are labile to both heat and light. Examination of the different series of results obtained indicated that the absorption bands at 410–460 $m\mu$, 560 and 664 $m\mu$ are due to separate chemical entities, because they did not appear and disappear together. It is probable, however, that the material responsible for the 664 $m\mu$ band is the precursor of the other substances, since it was always produced first and as the 664 $m\mu$ maximum diminished so the other absorption bands became more prominent.

When solutions of retinene in ethanol are mixed with phosphoric acid, a series of colour changes occurs. The solutions are blue immediately after mixing, and pass through violet and red stages before becoming brown. Attempts made to extract fractions of the coloured materials at different stages by shaking with common organic solvents met with little success. The coloured substances invariably remained in the acid phase. Dilution with ice and water before extraction produced a yellow chloroform extract, the absorption spectrum being flat and featureless, except for a maximum of low intensity at 300 $m\mu$. When this chloroform solution was treated with the SbCl_5 reagent a red solution was obtained, the absorption spectrum of which showed intense bands at 475 and 520 $m\mu$, and weak bands at 505 and 535 $m\mu$.

Additional observations The use of concentrated hydrochloric acid gave results similar to those obtained with phosphoric and sulphuric acids, though in this case the absorption spectra were not measured in detail. However, the same colour changes were noted, i.e. transient blue, passing through intense red to dirty brown, the sequence of changes being very much more rapid with hydrochloric acid than with either of the other acids.

Attempts to fractionate the different materials by chromatography failed, although both kieselguhr and alumina columns adsorbed the coloured products from the chloroform acid-vitamin A reaction mixture. The substances remained adsorbed in a

narrow zone at the top, irrespective of which organic solvent was used for development. It was found that the colour reactions of vitamin A and retinene with sulphuric acid were inhibited by dilution of the acid with water. In 50 and 80% (v/v) concentrations, sulphuric acid gives no coloration with vitamin A or retinene.

vitamin A molecule, which undergoes polarization and forms positively charged strongly resonating structures.

As a first possibility, Meunier (1942) suggests that the hydroxyl group of vitamin A attaches itself to the acid earth leaving a positively charged carbonium ion. With this type of structure the positive charge

Table 1 Comparison of the action spectra of Granit's modulators and the absorption spectra of vitamin A and retinene dissolved in strongly acidic solvents

(Wavelengths of maximum absorption, λ_{\max} , in $m\mu$.)

Rhodopsin	—	500	—	—	—	—
Iodopsin	—	—	—	560	—	—
Domimotors	—	500	—	560	—	—
Modulators	450-465	500	520-530	—	580-610	—
Vitamin A in conc. H_2SO_4	465	—	520-530	—	590	620
Vitamin A in conc. H_3PO_4	440-480	—	520	—	—	620
Retinene in conc. H_2SO_4	440-460	—	520	560	—	664
Retinene in conc. H_3PO_4	470	500	—	550	590	664

DISCUSSION

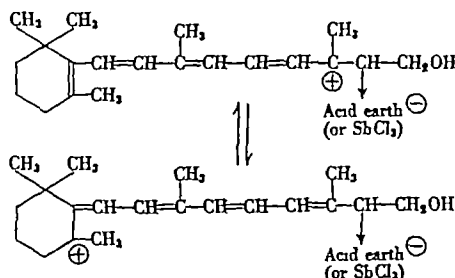
The absorption spectra observed when vitamin A or retinene is dissolved in concentrated sulphuric acid or syrupy phosphoric acid bear a striking resemblance to the action spectra of Granit's modulators (see Granit, 1947). This similarity is obvious from the summary of both sets of results given in Table 1.

Granit's modulator maxima fall roughly into three spectral ranges, and the absorption maxima obtained by the mineral acid reactions also fall into similar groups. Some additions to Granit's maxima must be noted, e.g. the 560 and 550 $m\mu$ bands obtained from retinene.

There can be little doubt that the absorption bands obtained are characteristic of 'ionized' or 'halochromic' molecules, as has been postulated by Meunier (1942) for the blue colour obtained when vitamin A is treated with the antimony trichloride reagent. Vitamin A gives a similar blue colour when

would resonate through a system of five conjugated double bonds giving rise to the 620 $m\mu$ maximum.

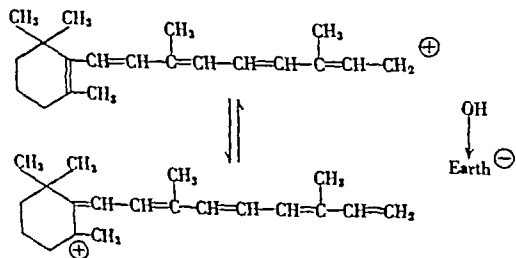
As a second mechanism Meunier postulated a polarization of the double bond nearest to the primary alcoholic grouping and resonance between the following limiting forms:



With this type of structure the positive charge would resonate through a system of four conjugated double bonds giving rise to the 590 $m\mu$ maximum. Meunier & Vinet (1947), carrying the procedure still further, suggest that resonance of a positive charge through two and three conjugated double bonds would give rise to maxima at 500 and 540 $m\mu$ respectively.

The absorption bands obtained with inorganic reagents are apparently narrower than those implied by Granit's action spectra, this difficulty will be discussed in a later paper.

The photopic receptor substances, presumably conjugated proteins, must be present at the cone surfaces, they must be relatively thermostable as otherwise there would be a spontaneous sensation of light (de Vries, 1948), and when detached from the protein moiety, the halochromic or ionized pro-



adsorbed on certain acid earths, e.g. montmorillonite, and Meunier (1942) suggested that the mechanism was similar to that of the antimony trichloride reaction. These acid earths (or antimony trichloride) possess incomplete electronic octets and are able to accept unshared electrons from the

thetic grouping derived from vitamin A or retinene would yield no materials not known to be present in the eye

CONCLUSIONS

The modulator analogues which have been obtained fulfil nearly all the requirements of the colour receptors whose presence is implied by Granit's work, e.g. during fading processes, the maxima fall at different rates, suggesting the presence, not of one product with several bands, but of several products with one maximum to each. The conditions under which these modulator analogues have been produced are obviously unphysiological.

If the problem posed by Granit's modulators is put in the form of a query whether or not the vitamin A-retinene oxidation-reduction system could possibly account for the phenomena observed, the answer is clearly in the affirmative. Which catalysts, old or new, must be invoked before the modulator

analogues can be obtained under less obviously unphysiological conditions is a matter for further research.

SUMMARY

1 The absorption spectra of vitamin A and of retinene₁ in concentrated sulphuric, phosphoric and hydrochloric acids have been studied. Sharp absorption bands characteristic of unstable ionized molecules were obtained.

2 The results show that vitamin A and retinene₁ can give rise *in vitro* to materials simulating the photopic modulators of Granit.

3 The hypothesis that the system vitamin A-retinene₁ is important in photopic as well as scotopic vision is strengthened.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance, and to Dr A. L. Stubbs and Dr F. D. Collins for help in the course of the work.

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Studies in Vitamin A

11 REACTIONS OF RETINENE₁ WITH AMINO COMPOUNDS

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(Received 11 February 1949)

Rhodopsin or visual purple is a conjugated protein obtained from dark adapted retinæ of many species. The prosthetic group is responsible for the colour (λ_{\max} 500 m μ) and photosensitivity, and is derived from vitamin A or its aldehyde retinene₁. No explanation has so far been advanced to account satisfactorily for the displacement of λ_{\max} from either 328 m μ (vitamin A) or 370–390 m μ (retinene₁) to 500 m μ .

The decomposition product of rhodopsin known

as indicator yellow shows λ_{\max} 440 m μ in acid solution and 365 m μ in alkaline solution. Neither the pH sensitivity nor the 440 m μ maximum has been properly accounted for.

The interaction of purified retinene₁ with amino compounds throws some light on these problems. A preliminary account of the work (Ball, Collins, Morton & Stubbs, 1948) has appeared, and the present paper carries the study a stage further.

EXPERIMENTAL

General procedure (a) A solution of crystalline retinene₁ in ethanol (about 10^{-6} M) was prepared so that the optical density (*E*) at 380–390 m μ . was 1.5–2.5, for a 1 cm layer. To the solution (2 ml.) was added an aqueous solution of amino compound (2 ml.) In some cases the latter solution was nearly saturated, and in all cases the concentration exceeded 10^{-3} M. To the mixture, 0.1N aqueous NaOH (2 ml.) was added, and the final mixture was left to stand at room temperature for 15–30 min. The absorption spectrum of the solution was then measured. Next, conc. HCl (1 drop) was added and the absorption spectrum redetermined, using a compensating cell containing water. The Beckman spectrophotometer was used throughout. In general, the alkaline solutions showed λ_{\max} 360–370 m μ . and the acidified solutions λ_{\max} 435–460 m μ .

General procedure (b) A number of amino compounds in which the NH₂ group is attached directly to the benzene ring produce a reddish colour with retinene₁ in the presence of acid. Pretreatment with alkali is not necessary, and λ_{\max} appears at 490–535 m μ . depending on the nature of the amino compound.

Conditions of interaction of retinene₂ and amino compounds The effect of varying the molecular ratio amino compound/retinene₁ has been investigated in the case of β alanine

RESULTS

A variety of compounds containing amino groups has been tried by procedure (a). Urea, formamide and dimethylamine significantly failed to shift the

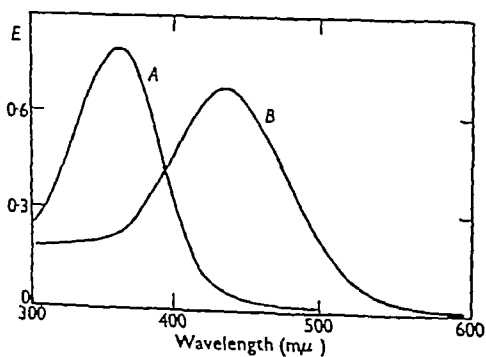


Fig 1 Absorption spectrum of retinene₁ plus methylamine (in excess) A, after standing (10 min.) in alkaline medium, B, subsequently acidified.

retinene₁ absorption spectrum. Simple amines and aliphatic amino acids, as well as tyrosine and tryptophan, reacted slowly with retinene₁ in alkaline solution giving a displacement of λ_{\max} from 380–390 to 360–370 m μ . On acidification a new band λ_{\max} 435–460 m μ . appeared (Figs 1 and 2).

The effect lacks specificity, since a variety of proteins show it, and the only difficulty is that instead of an orange yellow solution, an orange precipitate is sometimes formed (Table 1)

Aniline and various compounds related to it produce on the other hand a colour in acid solution immediately on mixing, no treatment with alkali

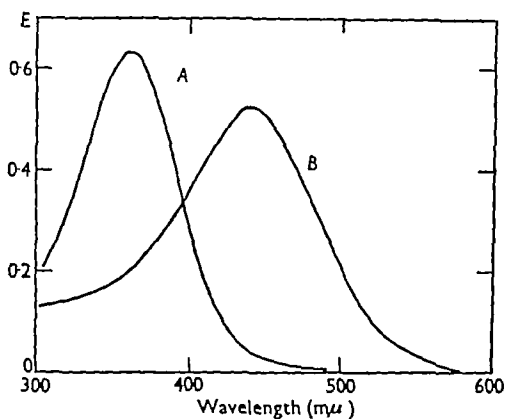


Fig 2 Absorption spectrum of retinene₁ plus β alanine (in excess) A, after standing (10 min.) in alkaline medium, B, after subsequent acidification. Retinene concentration 1.3×10^{-5} M, 1 cm cell

Table 1 Wavelengths of maximum absorption of mixtures of retinene₁ and amino compounds in aqueous ethanol

(The mixtures were first made alkaline and left to stand and then acidified. The amino compound was in considerable excess over retinene₁.)

Amino compound	λ_{\max} in alkali (m μ .)	λ_{\max} in acid (m μ .)
Methylamine	360–365	435
Dimethylamine	385–390	385–390 (no change)
Benzylamine	365	445
Urea	385–390	385–390 (no change)
Formamide	385–390	385–390 (no change)
Glycine	372	440–445
β Alanine	365	440
Serine	370	435–440
Isoleucine	370	450–455
Tyrosine	365	445
Tryptophan	360–365	445
Glutamic acid	375	435–440
Lysine	365	440–445
Arginine	365–370	460
Egg albumin	370	450
Peptone	365	440
Edestin	380	455
Trypsin	Solution not clear	440–450
Gelatin	360	440
Casein	360	Orange yellow precipitate
Zein	360–365	Orange yellow precipitate

being necessary (Fig 3, aniline, Fig 4, *p*-amino benzoic acid). This colour is due to a chromophore with λ_{\max} 490–535 m μ (Table 2)

Table 2 Wavelengths of maximum absorption of mixtures of retinene₁ and amino compounds, which result in visible colour in acid solutions without preliminary treatment with alkali

Amino compound	λ_{\max} in acid solution (m μ)
Aniline	490-500
Methylaniline	490
Benzylaniline	490
Dimethylaniline	No colour produced
1 Naphthylamine	500
2 Naphthylamine	515
<i>p</i> Toluidine	500
<i>o</i> Aminophenol	505
<i>p</i> Aminobenzoic acid	530
Anthranilic acid	520
<i>p</i> Aminobenzenesulphonamide	520
Diphenylamine	535

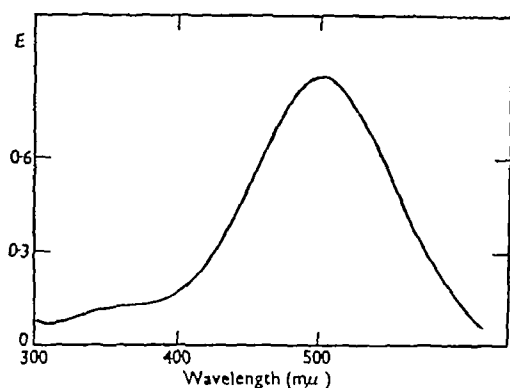


Fig 3 Absorption spectrum of retinene₁ plus aniline (in excess) in acid solution, retinene concentration 1.58×10^{-5} M, 1 cm cell.

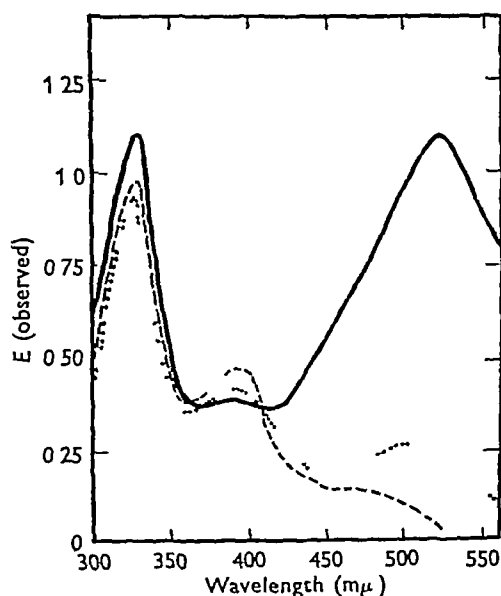


Fig 4 Absorption spectrum of retinene₁ plus *p* aminobenzoic acid acidified, —, solution examined at once, - - -, after 6 hr exposure to diffused daylight, - · - · -, after 10 hr exposure to daylight

In procedure (a) it is necessary to have the amino compound in excess over the retinene₁ if the change in λ_{\max} from 380-390 to 360-370 m μ in alkali is to be effected quickly (e.g. in 10 min). The molecular ratio of, for example, β alanine/retinene needs to be about 200, but a smaller excess will show the displacement in 48 hr (Table 3). Even in quite alkaline media (0.033 M-NaOH) there is no shift in λ_{\max} when β alanine and retinene₁ are mixed in equimolecular proportions. With excess of β alanine the reaction occurs readily in 0.033 M-NaOH, but very slowly in neutral solution (measured pH 6.98), a small shift only being obtained after 48 hr.

Table 3 Interaction of retinene₁ and β alanine in 0.033 M NaOH and ethanol

(The mixture was left to stand and then acidified with one drop conc. HCl, retinene, 1.3×10^{-5} M, β alanine in excess)

Molar ratio β alanine/retinene	Alkaline solution λ_{\max} (m μ)	Acidified solution λ_{\max} (m μ)	Time, NaOH solution was left to stand
0.5	385	385	48 hr
1	375	385	48 hr
30	370	395	48 hr
60	390	395	10 min
60	365	407	48 hr
120	370	410-415	10 min
180	370	425	10 min
240	365	430	10 min
480	365	440	10 min
600	365	440	10 min

Retinene₁ (33 mg) and β alanine (5 mg) were dissolved in ethanol (2 ml), 0.1 N ethanolic NaOH (2 ml) added, the mixture allowed to stand for several hours in the dark and then acidified with concentrated hydrochloric acid. The solution became dark red (λ_{\max} 440-450 m μ , using a small portion diluted with ethanol). If one drop of the red solution was diluted with water, the colour disappeared and λ_{\max} occurred at about 390 m μ . The solute obtained by removal of solvent *in vacuo* from the red solution was insoluble in light petroleum, but soluble in chloroform (λ_{\max} 465 m μ). The red solution showed no spectroscopic evidence of free retinene in appreciable proportion, although the retinene₁ and β alanine were used in the molecular ratio 2:1.

Those amino compounds which result in λ_{\max} of about 500 m μ permit an approximately quantitative study of colour intensity (E at 490-500 m μ) in relation to concentration of reactants and combining proportions. This is possible for the 500 m μ chromogen because retinene₁ has no appreciable absorption at that wavelength, whereas the overlapping of the 440 m μ absorption and retinene₁ absorption is a serious complication. Benzylaniline, which gives λ_{\max} 490 m μ although it has only one available hydrogen, was used for the stoichiometric test. Table 4 illustrates the results. The assumption

Table 4 *Interaction of retinene₁ and benzylaniline in acid solution*

$$K = \frac{[\text{resultant}]}{[\text{retinene}] [\text{amine}]}$$

Retinene concentration ($M \times 10^{-5}$)	Benzylaniline concentration ($M \times 10^{-5}$)	$E_{490m\mu}$ (corr)	K
1.59	1.59	0.139	1.85×10^4
1.59	3.18	0.246	1.95×10^4
1.59	7.95	0.392	1.68×10^4
1.59	15.9	0.516	1.69×10^4
1.59	Large excess	0.723	—

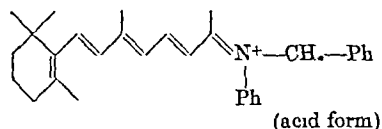
that one molecule of retinene reacts with two molecules of benzylaniline does not lead to an equilibrium constant, but a reasonably good constant is obtained on the basis of one molecule of retinene reacting with one molecule of benzylaniline

DISCUSSION

To some extent the large excess of amino compounds needed in these experiments reflects the fact that very high dilution is needed for the spectrophotometry. The evidence, however, points to the interaction of two molecules of retinene₁ with one molecule of methylamine, glycine, etc., since one molecule of retinene₁ with one molecule of aliphatic amine could scarcely give the indicator yellow type of spectrum. This implies that conjugated proteins resembling and including indicator yellow itself possess two retinene₁ molecules attached to the same amino nitrogen atom. The molecular extinction coefficients of the methylamine derivative are about 53,000 and 45,600 in alkaline and acid media respectively (calculated/mole of retinene₁). The β alanine derivative shows ' ϵ ' values of about 49,000 and 40,000. As the ϵ values for vitamin A and retinene₁ are respectively 50,000 and about 40,000,

the chromophoric change is confined to a displacement of wavelength.

The structural possibilities for the interaction of retinene₁ and *N*-benzylaniline are limited and the structure shown here is the most plausible one. There



are serious objections against the assumption that the results of Table 1 suggest that rhodopsin contains a nitrogen atom attached both to an aromatic ring and a retinene type of molecule—especially since that would imply a different linkage in indicator yellow from that in rhodopsin. Indeed it is not necessarily true that rhodopsin is a retinene, rather than a vitamin A derivative, although indicator yellow is certainly derived structurally from retinene₁. These, and other points concerning indicator yellow obtained from retinase, will be discussed in a later paper.

SUMMARY

1 Retinene₁, left to stand for a short time in alkaline solution with excess of many amino compounds and proteins, exhibits the absorption spectrum of alkaline indicator yellow, and on acidification shows that of acid indicator yellow.

2 Retinene₁, treated in acid solution with aniline and similar compounds, gives a red solution with λ_{\max} about 500 $m\mu$. This shows that retinene can give rise to a rhodopsin like chromophore although in a different way from rhodopsin.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance. One of us (F.D.C.) is the holder of a New Zealand National Research Scholarship.

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Studies in Vitamin A

12 WHALE-LIVER OIL ANALYSIS PREPARATION OF KITOL ESTERS

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(Received 9 March 1949)

Whale liver oil is valued for its vitamin A content, but this has so far been difficult to determine. Indeed, there has been no satisfactory alternative to the biological assay, which is itself of limited utility, if only because the fiducial limits of error ($P = 0.95$), using eighty rats, may often be no better than 70–140% of the estimated potency. Whale liver oil is, moreover, a very variable material. The crude oil, obtained by processing livers at sea or in Antarctic stations, usually contains phospholipins, water, free fatty acids and vitamin A decomposition products, as well as other impurities. In addition there is kitol, a substance not known to occur in comparable quantity in any other fat. The refined whale-liver oils of commerce also vary considerably. Quite apart from the fairly wide range of vitamin A content in crude oils, the refined oils are obtained in different ways and the resulting products are better described as concentrates. In some, the vitamin A may exist mainly in ester form, and in others it may be predominantly present as the free alcohol. The concentrates are often relatively free from oxidation products derived from vitamin A, but they are rarely, if ever, free from kitol. Anhydrovitamin A (almost certainly an artifact) is also present to a variable extent in whale liver oil samples.

Some decomposition of vitamin is perhaps unavoidable as a result of the difficulties inherent in working up in factory ships large amounts of liver containing only a low percentage of fat. Opinions have differed as to whether kitol is or is not present in the liver of the living animal. In any case the presence of anhydrovitamin A, of oxidation products and of kitol, gives rise to serious difficulties when the ultraviolet absorption or the antimony trichloride colour test is used to determine vitamin A in whale-liver oils or concentrates. Among the interfering substances, kitol is specially interesting because its biogenesis and possible function await elucidation.

Edisbury, Morgan & Morton (1935) found that, compared with fish-liver oils, whale-liver oils and concentrates exhibited anomalous ultraviolet absorption and abnormal colour tests, and Edisbury, Morton, Pritchard & Wilkinson (1937) described a fractionation of unsaponifiable extracts by partitioning between light petroleum and 83% (v/v)

aqueous ethanol. The fraction soluble in 83% ethanol contained nearly all the vitamin A, whilst the light petroleum soluble fraction showed an ultra-violet absorption curve with a maximum at 285–290 $m\mu$ and gave a purple rather than a blue colour with the antimony trichloride reagent (λ_{\max} 594 and 496 $m\mu$). The $E_{1\%}^{1\text{cm}}$ (285 $m\mu$) value was raised to 240 (which would to day be regarded as indicating 30–35% kitol), and the biological assay indicated about 17,900 i.u./g, corresponding possibly to about 0.5% of residual vitamin A or a very low intrinsic potency for the new substance.

Embree & Shantz (1943) subjected to molecular distillation a de-sterolized whale liver oil concentrate dissolved in a maize oil fraction and eliminated vitamin A at 150°. The residue yielded a non-saponifiable fraction, which, after chromatography on alumina, yielded a resin exhibiting λ_{\max} 290 $m\mu$, $E_{1\%}^{1\text{cm}}$ 580, and in the colour test showing maxima at 428, 505 and 580 $m\mu$. They named the main constituent kitol. When the material was again subjected to molecular distillation elimination began at 175° and became maximal at 225°. The product was, however, less pure than before, because the kitol had decomposed, yielding a certain amount of vitamin A.

Embree & Shantz (1943) reported the following composition and characteristic properties of kitol. Mol. wt. 575. Formula, $\text{C}_{40}\text{H}_{58}(\text{OH})_2$. Yield of vitamin A by thermal decomposition 1 mol vitamin/mol kitol. $[\alpha]_{\text{D}}^{25} -1.35$. λ_{\max} 286 $m\mu$, $E_{1\%}^{1\text{cm}}$ 580.

Baxter, Clough, Kascher & Robeson (1947) obtained a similar product with $E_{1\%}^{1\text{cm}}$ (290 $m\mu$) 586, and from it obtained kitol crystals (m.p. 88–90°) after first obtaining a crystalline di-*p*-phenylazo benzoate. The pure kitol gave $E_{1\%}^{1\text{cm}}$ (290 $m\mu$) 707 and the formula $\text{C}_{40}\text{H}_{58}(\text{OH})_2$ was confirmed. Geometric isomerism complicated the work on kitol esters. It is thus clear that kitol is a di(vitamin A), but its structure has not been elucidated and it cannot be 'depolymerized' by any method short of pyrogenic decomposition.

The present paper is concerned with (a) gaining further information about kitol, in particular whether it is or is not an artifact, and (b) the solution of the problem of analysing whale liver oils for

vitamin A The background of (b) is discussed later (p 314)

Hickman (1943) has suggested that kitol is a detoxication product in the sense that an 'excessive' accumulation of vitamin A is prevented when the inactive dimeride is formed in the whale liver This is a legitimate enough speculation, but even its narrower implications have yet to be studied Thus, the kitol/vitamin A ratio might perhaps vary with the potency of the oil and perhaps with the age of the whale On the other hand, if kitol were found to be a normal constituent of whale blood and milk the suggestion would need to be reconsidered

EXPERIMENTAL

Whale milk

Kitol

Only one sample of milk, and that from a fin whale, has reached us It contained 18% fat (0.7% of unsaponifiable matter), approx. 380 i.u. vitamin A/100 g. milk and showed ultraviolet absorption consistent with the presence of some kitol (Fig 1) Examination for kitol of milks from sperm whales and blue whales is desirable, but material is not easily obtained.

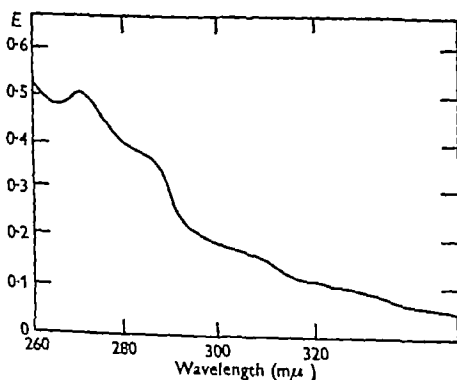


Fig 1 Ultraviolet absorption spectrum of fin whale milk lipid, 1.75% in cyclohexane, 1 cm

Whale liver oil

The crude commercial oil is usually very dark and highly viscous Our early attempts to prepare pure kitol esters were hindered by the presence of free fatty acids and phospholipins in the material subjected to chromatographic adsorption. Much better results were obtained with acetone soluble fractions, particularly those obtained in the laboratory from preserved liver The ease with which phospholipins dissolve in light petroleum is a disadvantage, because they tend to accompany kitol esters in chromatography The following section illustrates the difficulty

Phospholipins of whale liver oil Crude sperm whale-liver oil (2.212 g) was extracted many times with acetone, and the insoluble residue (0.766 g) was dried in a current of CO_2 . The residue, dissolved in light petroleum, was poured on a column (30 x 1 cm) of full strength alumina. The chromatogram was developed with light petroleum. The eluate con-

tained 0.41 g of material giving no colour with SbCl_5 reagent and only weak general absorption in the ultraviolet with a faint inflexion at 275 mμ.

Further development with light petroleum ether mixture (4:1) gave 0.097 g of a pale yellow oil, λ_{max} 290 mμ, $E_{1\text{cm}}^{1\%}$ 121, and ether ethanol mixtures gave 0.153 g of pale oil, λ_{max} 295 mμ, $E_{1\text{cm}}^{1\%}$ 157. Ether extracted most of the residual material. There seems to be in whale liver oil a substance of which kitol is a component and possessing an $E_{1\text{cm}}^{1\%}$ value near 160.

By dissolving crude sperm whale liver oil in light petroleum and adding acetone, a phospholipin fraction is thrown out of solution. The insoluble material may be redissolved in petroleum and reprecipitated by means of acetone. If this is repeated several times the material adsorbing selectively at 290 mμ can be removed. The residual phospholipin shows a feeble inflexion at 275 mμ, and after chromatography the various fractions exhibit $E_{1\text{cm}}^{1\%}$ (275 mμ) 23.6–32.4. The petroleum soluble phospholipin (0.61 g) yielded 0.063 g of unsaponifiable matter with λ_{max} 290 mμ, $E_{1\text{cm}}^{1\%}$ 40. Most of the ultraviolet absorption of the phospholipin was thus due to saponifiable constituents, but a small amount of material which does not give a colour with the SbCl_5 reagent is present and remains to be accounted for.

Although the main point of this section is to show the need for eliminating phospholipins it is clear that they are likely to repay further study. It is possible that kitol may play a part in glyceride phospholipin exchanges. It may well be that an investigation of the lipids of the absorptive portions of the whale intestinal tract would be worth while, in spite of the formidable difficulties of collecting and preserving the material.

Frozen whale liver as a source of oil

Large portions (50 kg) of fresh livers of sperm whale (*Physeter catodon*, syn *Physeter macrocephalus*) and blue whale (*Balaenoptera musculus*) were frozen right through in the Antarctic. The liver was brought to England in the ship's refrigerator and thereafter kept in a commercial cold store, and portions were chopped off as required.

Blue whale liver A portion (approx. 2 kg) of liver was thawed, sliced and minced. The mince was extracted several times with cold acetone. The combined, concentrated, acetone extract was mixed with water and the fat extracted with light petroleum. The combined extracts were washed with water, dried over Na_2SO_4 and the solvent was removed. The product was 40 g of yellow oil which became brown on standing. sp gr about 0.95, I_d val 163.4, saponification equiv 330.8. The ultraviolet absorption curve showed λ_{max} 328 mμ, $E_{1\text{cm}}^{1\%}$ 36.7 (gross), 29.8 after correction according to the procedure of Morton & Stubbs (1946). The curve (Fig 2) was not very abnormal compared with that of a pure vitamin A ester. The kitol content was not high.

The liver debris was ground and re-extracted with ether ethanol mixture (3:2 v/v). The filtered extract was diluted with water and re-extracted with ether. The solute (2 g) contained very little vitamin A or kitol, but some decomposition products were present.

The oil from this sample of frozen blue whale liver contained much less kitol than vitamin A, and there was relatively little interference with spectrophotometric determinations of vitamin A.

Sperm whale liver The oil obtained by extraction of minced liver with cold acetone was light brown in colour but darkened on standing, I_2 val 149.8, saponification equiv 378.5. The ultraviolet absorption curve showed a very flat maximum near $310\text{ m}\mu$, $E_{1\text{ cm}}^{1\%}$ (328 $\text{m}\mu$), 193.5, (286 $\text{m}\mu$) 122.9 (gross) (Fig. 3). The oil contained larger absolute amounts of vitamin A and kitol than that from blue whale liver, and indeed a rough approximation indicated some 16% of vitamin A esters and perhaps 20% of kitol esters. The liver debris again gave a small yield of residual ether soluble material.

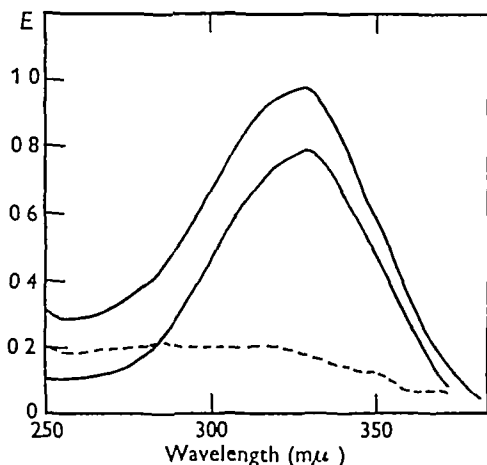


Fig. 2 Ultraviolet absorption spectrum of cold acetone extract of blue whale liver $E_{1\text{ cm}}^{1\%}$ (328 $\text{m}\mu$) 36.7 (gross), 29.8 (corrected), solvent, cyclohexane. Upper curve, observed, lower curve, corrected, broken line, subtraction curve.

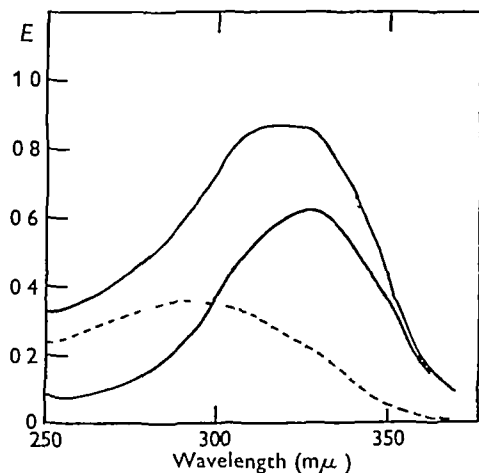


Fig. 3 Ultraviolet absorption spectrum of cold acetone extract of sperm whale liver $E_{1\text{ cm}}^{1\%}$ (328 $\text{m}\mu$) 193.5 (gross), 141.5 (corrected), solvent, cyclohexane. Upper curve, observed, lower curve, corrected, broken line, subtraction curve.

The oil from this sample of sperm whale liver was thus a good source both of vitamin A and kitol, but, as the latter was present in large amount, con-

siderable interference occurred in the spectrophotometric determinations for vitamin A.

The experiments on well-preserved frozen livers suggest that in commercial whale liver oils the presence of vitamin A decomposition products is a more serious analytical obstacle than the presence of kitol. This point is reinforced by the results of tests on salted minced whale livers. It is not necessary to go into all the experimental details, but it may be said that the oil samples obtained by various methods of extraction were fairly uniform, the $E_{1\text{ cm}}^{1\%}$ (328 $\text{m}\mu$) values (uncorrected) varying from 37 to 60. The percentage of unsaponifiable matter was about 25. Anticipating the discussion of the analytical problem (see p. 314), the fraction of the gross absorption at 328 $\text{m}\mu$ due to vitamin A is about two thirds and a conversion factor of 1200 is roughly correct. Attention is drawn at this stage to the fact that the oils obtained in the laboratory from preserved whale livers show the type of absorption spectrum to be expected from the summation of contributions due to kitol and vitamin A. From the point of view of analysis for vitamin A, the interference due to kitol is serious, but in commercial oils there is the additional difficulty of substantial irrelevant absorption due to artifacts.

Fractionation of whale liver oils

Sand-column technique Chevallier, Manuel & Faubert (1941) dispersed fish liver oil on sand prior to extraction with solvents. The following experiment was carried out on the lines of their work. Whale liver oil concentrate (0.05 g) was dissolved in 10 ml redistilled ethyl ether, and the solution poured on to a column of fine, dry, silver sand (2 x 20 cm). After allowing the solution to seep into the sand the ether was removed by a brisk upward current of N_2 . The column was then washed with successive portions of solvent, with the results shown in Table 1. It was clear that 80% ethanol, if used in sufficient quantity, could extract anhydrovitamin A and vitamin A, leaving kitol behind.

Table 1 Elution of whale liver oil concentrate dispersed on sand

Eluting solvent Ethanol %	Volume (ml)	λ_{max} (μm)	$E_{1\text{ cm}}^{1\%}$ *	Vitamin A $E_{1\text{ cm}}^{1\%} \cdot 328 \mu\text{m}$	
				Gross	Corrected
(a) 0.05 g oil used					
70	270	328	—	42.4	33.6
80	540	328	—	50.8	26.6
90	90	292	17.6	—	—
100	90	290	25.3	—	—
(b) 0.05 g oil used					
60	90†	328	—	19.04	7.84
70	90†	328	—	9.65	6.44
80	90†	328	—	19.04	15.96
90	90	328	—	38.8	30.1
100	90	295	53.2	—	—

* Calculated on weight of original oil.

† Solvent passed through column five times.

A further experiment was tried on a larger scale and sand was not used. 0.5105 g of 'oil' was boiled with 80% ethanol, the solution cooled and the supernatant liquor decanted. The 80% ethanol insoluble fraction (0.3075 g) showed λ_{\max} 310–313 $m\mu$ and contained about 4% vitamin A and 14% kitol. The soluble fraction (0.199 g) showed λ_{\max} 328 $m\mu$ and contained some 3% of vitamin A contaminated by anhydrovitamin A and free kitol. The 'insoluble' fraction was repeatedly washed with hot 80% ethanol, cooled and the supernatant liquid decanted. The residue showed λ_{\max} 295 $m\mu$, $E_{1\text{ cm}}^{1\%}$ 125, corresponding with some 18% of kitol or about 36% kitol esters. This fraction (0.16 g) was boiled with considerable excess of ethanolic KOH and 0.1044 g unsaponified residue was obtained containing about 4% vitamin A and 23% 'free' kitol. This material was chromatographed on full strength alumina (Spence, Grade 0) using light petroleum as the developing solvent. The various fractions were examined spectrophotometrically (Table 2). If $E_{1\text{ cm}}^{1\%}$ (286 $m\mu$) = 700 for pure kitol the material from the top zone contained 84% kitol (cf Fig. 4).

Table 2 Chromatography of 0.08 g of unsaponifiable material from the fraction of whale liver oil concentrate insoluble in 80% ethanol

Zones	Wt obtained (g)	λ_{\max} ($m\mu$)	$E_{1\text{ cm}}^{1\%}$ (286 $m\mu$)	$E_{1\text{ cm}}^{1\%}$ (286 $m\mu$) with respect to unsaponifiable material
Top	0.0105	286	588	77.20
Second	0.0096	286	317.1	39.63
Third	0.0564	325	21.3	14.90
Fourth	0.0019	—	—	0.75
—	0.0784	—	—	132.48

The foregoing experiments suffer from the disadvantage of being on a very small scale. In an attempt to overcome this, 50 g of oil were heated with absolute ethanol (1 l) until the solution became clear, and water (250 ml) was then added and the solution cooled. The mixture was then put through a Sharples centrifuge. The soluble portion showed λ_{\max} 326.5 $m\mu$ and contained most of the vitamin A and anhydrovitamin A. The insoluble portion was repeatedly extracted with small amounts of ethanol. The residue was chromatographed in light petroleum solution on full strength alumina. After several chromatographic separations it became clear that under these conditions fractions showing λ_{\max} 286 $m\mu$, $E_{1\text{ cm}}^{1\%}$ 166 were the best obtainable.

Chromatography of whole oil on a small scale gave two main kitol fractions, λ_{\max} 286–290 $m\mu$, $E_{1\text{ cm}}^{1\%}$ about 130 and 315, respectively, and the following decreasing order of adsorbability was indicated: free kitol, free vitamin A, kitol esters, vitamin A esters, anhydrovitamin A. Working on a 20–25 g scale, fractions showing $E_{1\text{ cm}}^{1\%}$ about 160 at 295 $m\mu$ were regularly obtained, but they were not free from vitamin A. One such fraction was chromatographed

on weakened alumina (Spence, Grade 0, stirred with 10% (w/w) water). A fraction containing practically all the vitamin A was quickly obtained when the column was developed with light petroleum and was followed by a fraction showing $E_{1\text{ cm}}^{1\%}$ 367.5 at 286 $m\mu$ and a further fraction exhibiting $E_{1\text{ cm}}^{1\%}$ 250 at 280 $m\mu$.

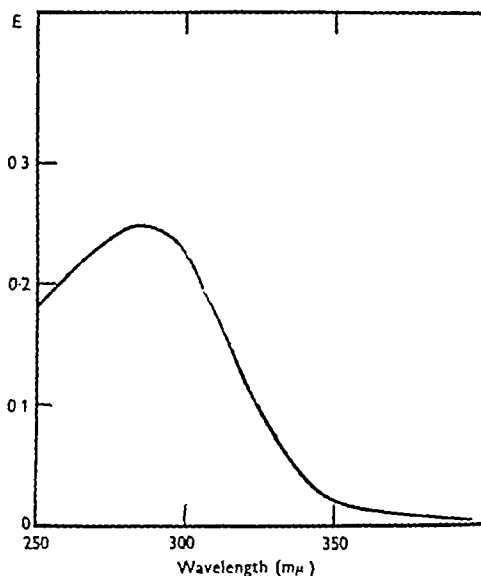


Fig. 4 Ultraviolet absorption spectrum of portion of whale-liver oil insoluble in 80% (v/v) aqueous ethanol. Unsaponifiable fraction chromatographed, strongly adsorbed zone at top of column, $E_{1\text{ cm}}^{1\%}$ (286 $m\mu$) 588 in cyclohexane.

Attempts to crystallize kitol esters from crude preparations in acetone at low temperatures gave products of λ_{\max} 280–286 $m\mu$, $E_{1\text{ cm}}^{1\%}$ 160. Chromatography on '5% weakened' alumina gave two fractions $E_{1\text{ cm}}^{1\%}$ 168 and 349.2, respectively.

Chromatography of cold acetone extract of frozen sperm whale liver

A portion of oil (4.18 g), dissolved in light petroleum (25 ml), was put through a column of full strength alumina and developed first with light petroleum (1 l) and then with light petroleum containing increasing amounts of acetone. The eluates (1–7) were examined spectroscopically with the following results: (1) Light brown oil, discarded. (2) λ_{\max} 328 $m\mu$, $E_{1\text{ cm}}^{1\%}$ 254, some anhydrovitamin A was present. (3) λ_{\max} 328 $m\mu$, $E_{1\text{ cm}}^{1\%}$ 282, absorption curve practically normal for vitamin A. (4) λ_{\max} 290–295 $m\mu$, $E_{1\text{ cm}}^{1\%}$ (286 $m\mu$) 253, gave no vitamin A band at 620 $m\mu$ in the colour test. (5) Contained some vitamin A alcohol, $E_{1\text{ cm}}^{1\%}$ (328 $m\mu$) 82. (6) λ_{\max} 328 $m\mu$, $E_{1\text{ cm}}^{1\%}$ 152, practically normal vitamin A curve (free alcohol). (7) Brown portion of column cut out, eluted with ether, fraction was mainly kitol (free), $E_{1\text{ cm}}^{1\%}$ (286 $m\mu$) 152.

Fraction 4 was rechromatographed on full strength alumina and developed with light petroleum. Two main zones appeared and three portions were cut out from the middle of the lower zone. All three were eluted with ether. The ether

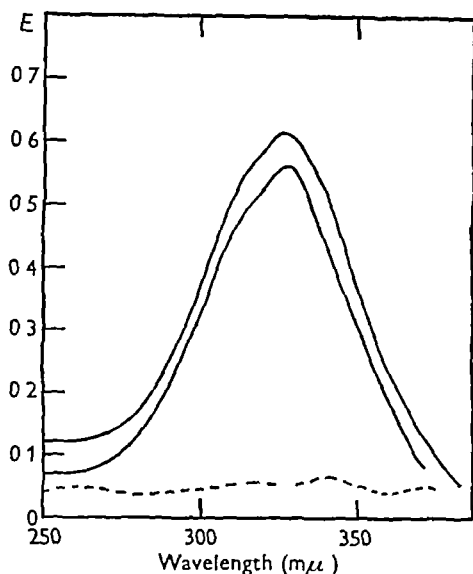


Fig 5 Chromatography of phospholipin free, neutral, sperm whale liver oil. Ultraviolet absorption spectrum of vitamin A ester fraction $E_{1\text{ cm}}^{1\%}$ (328 $m\mu$) 305 (gross), 280 (corrected) [or 112.8 (corrected) with respect to oil], solvent, cyclohexane. Upper curve, observed, lower curve, corrected, broken line, subtraction curve.

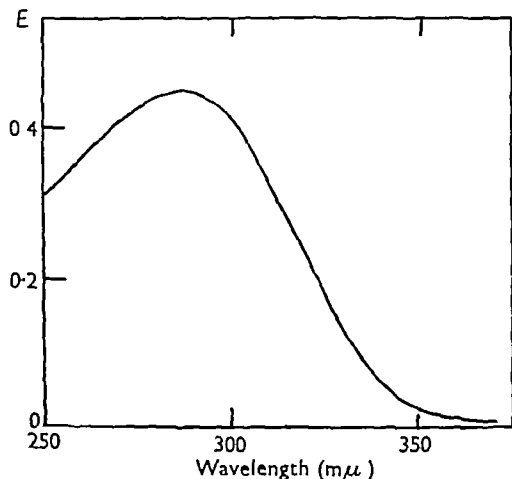


Fig 6 Ultraviolet absorption spectrum of kitol ester fraction, 0.0013% in cyclohexane, 1 cm $E_{1\text{ cm}}^{1\%}$ (286 $m\mu$) 346

was removed and the weighed residues were tested in cyclohexane. The absorption curves were very flat over the range 286-290 $m\mu$. and $E_{1\text{ cm}}^{1\%}$ values of 166, 343 and 353 were obtained.

A large number of experiments on sperm whale liver oil indicated that in seeking to prepare pure kitol esters it is

desirable to use material free from phospholipins or free fatty acids. The following method is perhaps the simplest. To sperm whale liver oil acetone is added and, after allowing phospholipins to settle out, the solution is filtered through a bed of kieselguhr mixed with solid NaHCO_3 (used as a fine powder). The filtrate is diluted with water and extracted with light petroleum, washed with water, dried over Na_2SO_4 , filtered and the solvent evaporated. The oil (approx 10 g) is dissolved in light petroleum and chromatographed on alumina weakened by addition of 10% (w/w) of water. Light petroleum is used at first to develop the chromatogram (Fig 5). The eluate is periodically tested with the SbCl_5 reagent. When the vitamin A colour test becomes weak, development is continued with light petroleum containing 5% ether (v/v). When kitol esters begin to be eluted a purple colour will be obtained with the SbCl_5 reagent. In two such experiments fractions showing $E_{1\text{ cm}}^{1\%}$ (295 $m\mu$) 346 (Fig 6) were obtained weighing 0.85 and 0.52 g respectively.

The best preparations of kitol (esterified) were all in agreement with the figure of $E_{1\text{ cm}}^{1\%}$ about 350 as the highest attainable.

Saponification of kitol esters

The results of attempts to prepare pure kitol by saponifying the esters and extracting the unsaponifiable fraction were erratic and rather disappointing. Thus one ester preparation

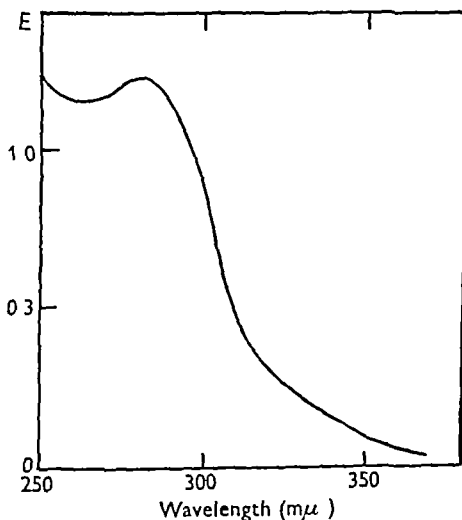


Fig 7 Ultraviolet absorption spectrum of kitol ester fraction, unsaponifiable matter after two treatments with alkali, $E_{1\text{ cm}}^{1\%}$ (286 $m\mu$) 230

showing λ_{max} 286 $m\mu$, $E_{1\text{ cm}}^{1\%}$ 350 gave 54.8% of unsaponifiable matter with λ_{max} 280 $m\mu$, $E_{1\text{ cm}}^{1\%}$ 156, but the kitol had undoubtedly decomposed. The recovered acid amounted to 43% of the material used. Another good ester preparation, left to stand in the cold with ethanolic KOH for 48 hr, resisted saponification. On treatment with ethanolic KOH at the boiling point in a current of N_2 , the $E_{1\text{ cm}}^{1\%}$ value of the unsaponifiable fraction rose to 410, but fell to 230 (λ_{max} 280 $m\mu$) after retreatment with alkali for a longer time on the steam bath (Fig 7). The recovered acids (33%) had a mean mol wt of 284.

With another preparation the total recovered fatty acids accounted for 48.3% of the kitol ester fraction. The following illustrates some of the later experiments. 0.771 g kitol ester fraction, $E_{1\text{ cm}}^{1\%}$ (286 $m\mu$) 346 was dissolved in ethanol (10 ml.) and 40% aqueous KOH (4 ml.) was added. A little quinol was used as antioxidant. After boiling on the water bath until the solution was quite clear, the unsaponifiable material was extracted in the usual way and the fatty acids recovered from the soaps. The $E_{1\text{ cm}}^{1\%}$ value at 285 $m\mu$ had risen to 432 and 0.296 g of acids was recovered. Resaponification with sodium ethoxide (boiling for 15 min) raised the $E_{1\text{ cm}}^{1\%}$ value of the non saponifiable material to 620 and gave a further 0.125 g of acids.

If kitol has a mol. wt of 572 and an $E_{1\text{ cm}}^{1\%}$ (286 $m\mu$) of 700, then an $E_{1\text{ cm}}^{1\%}$ value of 350 for mixed kitol esters implies that these are diesters of acids with an average mol. wt of about 304.

Pyrogenic decomposition of kitol esters

When heated carefully, kitol esters darken in colour, and the product shows weakly a maximum at 620 $m\mu$. In the SbCl_3 colour test. Small quantities (0.02 g) heated electrically in capillary tubes showed no decomposition at temperatures below 200°. From 230 to 250° (4–5 min.) faint positive tests were obtained (SbCl_3 max. 612–620 $m\mu$), but the yield of vitamin A from the pyrogenic decomposition of the esters was poor. The special conditions of molecular distillation are clearly needed for good yields.

Administration of kitol esters to the avitaminotic rat

When given as 0.12 g in 1 ml. of coconut oil spread over 2 days and administered by stomach tube, kitol esters were neither converted into vitamin A nor appreciably stored in the liver 40 hr. after the second dose.

A chromatographic method for the determination of vitamin A in whale liver oils

The experiments leading to the isolation of a kitol ester fraction suggested a new approach to the analytical problem. The matter has a somewhat complicated history. At an early period in the history of the use of whale liver oils as a source of vitamin A a commercial concentrate was studied (Edisbury *et al.* 1935, 1937) which exhibited anomalous ultra violet absorption (λ_{max} about 295–305 $m\mu$) and an anomalous colour test as compared with fish liver

oils. It was found empirically that by measuring $E_{1\text{ cm}}^{1\%}$ (328 $m\mu$) and multiplying by 1200, estimates of potency in reasonably good agreement with the results of bioassays were obtained.

With the resumption of whaling after the end of the War it became necessary to determine vitamin A in crude oils extracted on board the factory ships. It speedily emerged in these laboratories, and in those of Lever Brothers and Unilever Ltd. at Port Sunlight, that neither the colour test nor the ultra-violet absorption was anomalous in a constant fashion, and the basis for the empirical use of a conversion factor of 1200 instead of 1600 (widely used for fish liver oils) disappeared. The correction procedure of Morton & Stubbs (1946) was often stretched beyond the limits of its utility by the magnitude of the irrelevant absorption. It had also become clear that the factor of 1600 was itself a reflexion of the fact that, on the average, fish liver oils exhibited some 11% or so of 'irrelevant' absorption and that a factor of 1800 was appropriate to preparations in which vitamin A was the only significantly absorbing entity (Morton & Stubbs, 1947). The problem of analysing whale liver oils thus resolved itself into the task of obtaining all the vitamin in a fraction (or fractions) free from other substances absorbing near 328 $m\mu$. The following experiments indicate how the problem has been solved.

Sperm whale liver oil (1.713 g) was dissolved in 15 ml light petroleum in a small conical flask and powdered NaHCO_3 (1 g) added, and the mixture thoroughly shaken. Weakened alumina was prepared by placing aluminium oxide (50 g, Spence, Grade 0) in a mortar, covering with light petroleum and adding water (5 ml.). The mixture was stirred thoroughly with a pestle and used to fill a tube 44 x 1 cm. A layer (1.5 cm. high) of powdered NaHCO_3 was put on top of the column. The oil in light petroleum was then poured on to the column without disturbing the sediment. The residual contents of the conical flask were washed with light petroleum several times and the solution poured on to the column. Light petroleum was used to develop the chromatogram. A light yellow band travelled down the column and the eluate was collected in 20 ml. portions, a drop from each of which was tested with the SbCl_3 reagent. The portions were sorted out into three groups (A) those giving a normal vitamin A colour test, (B) those giving a purple colour and, finally, (C) those obtained only when the developing solvent contained ether (10 or 20% by vol. in

Table 3 *Chromatographic separation of constituents of sperm whale liver oil*

Fractions	Wt recovered (g)	λ_{max} ($m\mu$)	(Wt of oil, 1.713 g)		$E_{1\text{ cm}}^{1\%}$ with respect to oil	SbCl_3 colour test (λ_{max})
			$E_{1\text{ cm}}^{1\%}$			
A	0.517	328	(328 $m\mu$) 316 gross 280 corr		(328 $m\mu$) 96 gross 84 corr	620 $m\mu$. (blue)
B	0.364	300	(300 $m\mu$) 218.5 gross (328 $m\mu$) 34 corr (286 $m\mu$) 233 corr		(328 $m\mu$) 7.2 corr (286 $m\mu$) 49.4	(purple), 495 $m\mu$, 505 $m\mu$, 580 $m\mu$.
C	0.064	280	(280 $m\mu$) 38 gross		—	(Red)

light petroleum) The solvent was removed from the three fractions and the residues weighed and subjected to spectroscopic analysis (Table 3)

It is evident from Table 3 that fraction *A* contained most of the vitamin A and was easily analysed, *B* contained most of the kitol, and *C* consisted mainly of decomposition products. A further experiment was carried out to try to improve the separation.

Sperm liver oil (0.998 g) and NaHCO_3 (0.5 g) were shaken with light petroleum and the solution chromatographed on weakened alumina as before. The eluate was collected in 5 ml portions and those which gave a normal vitamin A blue with the SbCl_3 reagent were bulked, the solvent removed and the residue weighed (0.282 g). This was made up to volume with cyclohexane and a measured portion was diluted for spectrophotometry. The absorption maximum occurred at 328 m μ with $E_{1\text{ cm}}^{1\%}$ 100.8 (gross) calculated on the weight

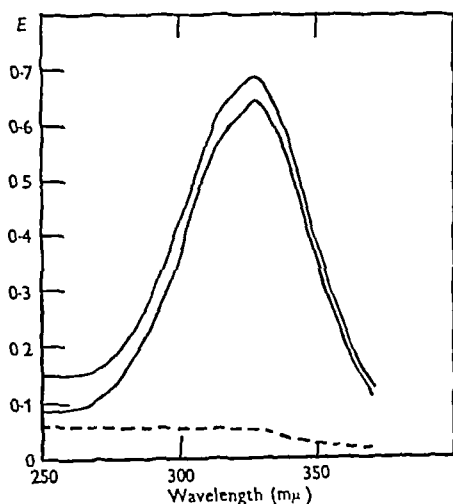


Fig. 8. Chromatographic analysis of sperm whale liver oil. Ultraviolet absorption spectrum of vitamin A ester fraction, $E_{1\text{ cm}}^{1\%}$ (328 m μ) 100.8 (gross), 94.4 (corrected) with respect to oil, solvent, cyclohexane. Upper curve, observed, lower curve, corrected, broken line, subtraction curve.

of oil used (Fig. 8). This value was reduced to 94.4 on applying the three point correction procedure for irrelevant absorption. The previous experiment gave $84.7 \pm 7.2 = 91.2$, but the vitamin was not all in the first fraction. It will be seen that by collecting the eluate in 5 ml portions, one drop of each of which is tested with SbCl_3 , a very clear cut separation can be achieved.

The procedure needs, however, to be subjected to a more severe test. Accordingly, a sample of low potency oil of inferior quality was tested.

Crude whale liver oil (0.2665 g) in light petroleum (20 ml) was chromatographed on a column of weakened alumina 15×1 cm and developed with light petroleum. The eluate was collected in 5 ml portions. The first few portions contained anhydrovitamin A (pale greenish yellow, blue colour with SbCl_3). The next four portions gave no colour with the

SbCl_3 reagent and were discarded. Subsequent portions contained vitamin A (esters) giving a normal colour test. Elution was continued until the solution was no longer chromogenic towards SbCl_3 . The column was then eluted with ether. The vitamin A fraction was made up to 50 ml with light petroleum and a measured portion diluted with cyclohexane so that the concentration was 0.0533% with respect to the original oil. It gave λ_{max} 328 m μ , $E_{1\text{ cm}}^{1\%}$ 8.74 (gross), 8.4 (corr.). The ether eluate was made up to 100 ml with light petroleum, and a measured portion diluted with cyclohexane to 0.02665% with respect to the original oil. It gave $E_{1\text{ cm}}^{1\%}$ (286 m μ) 12.5 and contained kitol esters together with decomposition products, but no vitamin A was detectable. (If at any time the percolation of solvent through the column becomes too slow, it is advisable to stir the adsorbent at the top with a glass rod.)

To test the method further a more complete analysis was made on another sample of crude oil. Crude whale liver oil (0.2 g) in light petroleum (20 ml) was poured on a column (15×1 cm) of weakened alumina, and the chromatogram developed with light petroleum followed by the same solvent containing increasing proportions of ether. The percolate was collected in 5 ml portions, 1 drop of each being tested with the SbCl_3 reagent.

The following fractions were obtained: (1) Anhydro vitamin A, discarded. (2) Vitamin A esters, 180 ml of eluate λ_{max} 328 m μ , $E_{1\text{ cm}}^{1\%}$ 14.6 (gross), 14.14 (corrected), E values calculated on weight of oil taken. (3) Kitol ester fraction, elution complete after using light petroleum containing 4, 8 and 12% ether. λ_{max} 290 m μ , $E_{1\text{ cm}}^{1\%}$ 8.125. (4) Fraction containing free vitamin A and some free kitol, λ_{max} 326 m μ , $E_{1\text{ cm}}^{1\%}$ 4.22 (gross), 3.02 (corr.), elution completed with 12, 16 and 20% ether in petrol. (5) Fraction eluted with ether, no vitamin A present as judged by colour test.

For this oil $E_{1\text{ cm}}^{1\%}$ (328 m μ , gross) was 28.7 and the net contribution due to vitamin A was $14.14 + 3.02 = 17.16$. The sample correction procedure of Morton & Stubbs (1946) gave 15.8—a figure which is significantly low.

Similar experiments have led to the following standard procedure.

Analysis of commercial whale liver oil

Adsorbent. Alumina (Spence, Grade 0) for adsorption (100 g) is made into a slurry with light petroleum in a glass mortar, and water (10 ml) is added and the whole mixed with a pestle for 5 min. The slurry is then poured into an adsorption tube and allowed to settle. The weakened adsorbent should be used the same day.

Column. The column of weakened alumina should be 12×1 cm if the weight of oil to be chromatographed is 0.2 g. The sample is dissolved in light petroleum (25 ml) and poured on to the column. A large bottle (15–20 l) is fitted with a rubber bung with two holes, through which tubes, each with a tap, are inserted, one is connected by a rubber tube to a bicycle pump and the other to a short piece of glass tubing in a rubber bung fitting the top of the

adsorption apparatus. Passage of solvent through the column is thus hastened by pressure from above, and percolation may be quite rapid (approx 2 ml/min). There should be room for a least 25 ml of solvent above the absorbent.

Development The chromatogram is developed first with light petroleum and the percolate collected in small test tubes graduated at 5 ml. One drop from each portion is tested with 0.5 ml of SbCl_3 reagent. The first few portions will give no colour and may be discarded. Then anhydrovitamin A will appear in the eluate, it gives a blue colour with the SbCl_3 reagent and, after a volume of 35–40 ml has passed through the column, no colour will appear. (The volume will, of course, vary somewhat from sample to sample.) Continued development with light petroleum results in the appearance of vitamin A esters in the eluate. The intensity of the blue colour obtained with one drop of successive portions of percolate will at first increase and finally decrease until no colour is given. By this time, 100–200 ml of liquid will have passed through the column. Development is continued at first with light petroleum containing 4% ether (by vol) and then with 8 and with 12% ether-petroleum mixtures, usually 50 ml portions at each strength. The percolate at this stage gives a purple colour with the SbCl_3 reagent and exhibits an ultraviolet absorption maximum near 285–290 $\text{m}\mu$. A small volume of eluate then appears which is practically non-chromogenic towards SbCl_3 . Development with ether-petroleum mixtures (12, 16, 20% by vol) is continued, and fractions are obtained giving blue and purple colours with SbCl_3 , indicating free vitamin A and free kitol respectively. The 5 ml portions of eluate are grouped and the separate fractions each made up to a definite volume and examined by photoelectric spectrophotometry.

Example. $E_{1\text{ cm}}^{1\%}$ (328 $\text{m}\mu$) 60.1 on the whole oil.

0.125 g examined as above

- (i) Anhydrovitamin A fraction $E_{1\text{ cm}}^{1\%}$ (370 $\text{m}\mu$) 1.6
- (ii) Vitamin A ester fraction, made up to 200 ml, diluted 5 ml./20 ml. cyclohexane $E_{1\text{ cm}}^{1\%}$ (328 $\text{m}\mu$) 37.9, no correction needed
- (iii) Kitol ester fraction, λ_{max} 285–290 $\text{m}\mu$ $E_{1\text{ cm}}^{1\%}$ 24.35
- (iv) Free vitamin A fraction, $E_{1\text{ cm}}^{1\%}$ (328 $\text{m}\mu$) approx 2.2 corr

Total $E_{1\text{ cm}}^{1\%}$ (328 $\text{m}\mu$) 40 (i.e. 37.9 + 2.1)

Estimated potency $40 \times 1800 = 72,000$ i.u./g

Separation of vitamin A from whale liver non-saponifiable matter by means of 50% ethanol

By washing the unsaponifiable fraction of whale liver oil with 50% (v/v) aqueous ethanol, free vitamin A is preferentially dissolved. Under certain circumstances this forms the basis of a useful analytical procedure.

Example A commercial whale liver oil concentrate (0.5 g, $E_{1\text{ cm}}^{1\%}$ (328 $\text{m}\mu$, 210 gross) was saponified, and the unsaponifiable fraction showed $E_{1\text{ cm}}^{1\%}$ 212.6 at 328 $\text{m}\mu$ (calculated on the wt of raw material). The absorption curve was, however, anomalous and there was evidence of considerable irrelevant absorption. The unsaponifiable matter was shaken well with 50% ethanol (100 ml.), and the supernatant liquid filtered carefully through a small bed of kieselguhr (filter aid). Six further washings (2×100 ml and 4×50 ml.) were filtered in the same way. The combined filtrate was made up to 500 ml (A). The flask and the filter aid were then washed repeatedly with small quantities of redistilled ether. The solvent was removed and the extract dried by treatment with ethanol in a current of N_2 on the water bath, the residue was dissolved in ethanol and the volume made up to 100 ml (B).

A measured volume of solution A was diluted with ethanol and showed λ_{max} 326 $\text{m}\mu$, $E_{1\text{ cm}}^{1\%}$ 135.8 (corrected by the Morton & Stubbs (1946) method). Solution B, suitably diluted, gave λ_{max} 287 $\text{m}\mu$, $E_{1\text{ cm}}^{1\%}$ 85 (E values calculated with respect to the original oil, see Fig. 9).

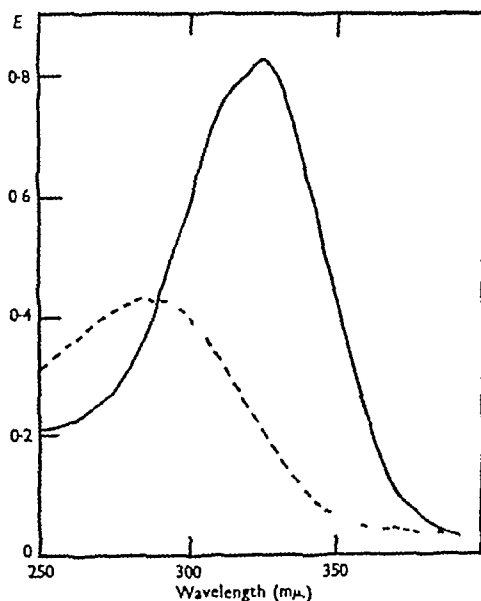


Fig. 9 Ultraviolet absorption spectrum — Portion of whale liver oil unsaponifiable material soluble in 50% (v/v) aqueous ethanol, $E_{1\text{ cm}}^{1\%}$ (326 $\text{m}\mu$) 164 (gross), 135.8 (corrected), calculated for original oil, solvent, ethanol. — Portion of unsaponifiable material insoluble in 50% ethanol, $E_{1\text{ cm}}^{1\%}$ (287 $\text{m}\mu$) 85, solvent ethanol.

Further trials showed that most, but not all, of the irrelevant absorption can be eliminated from whale-liver oil unsaponifiable fractions by extraction with 50% aqueous ethanol. The kitol fraction is relatively insoluble in this medium. Judged, however, by the strictest standards, the method is imperfect, because when an oil is contaminated with artifacts, as well as with kitol, a little extraneous

material absorbing at 326 $m\mu$ is liable to accompany the vitamin A alcohol. The test, however, often affords valuable confirmation (see Table 4)

Table 4 *Correction of absorption of a whale liver oil concentrate*

(Concentrate had $E_{1\text{cm}}^{1\%}$ (328 $m\mu$) 210 (gross))

	E (corr)	Vitamin A
Concentrate	141.4	* Assuming it to be all esterified
	128.8	* Assuming it to be free
Unaponifiable extract	130	* Assuming it to be free
50% ethanol extract	135.8	* Assuming it to be free

* The absorption curves for vitamin A alcohol and esters are not quite identical.

DISCUSSION

Numerous preparations from samples of whale liver oil have been obtained showing an absorption maximum at 285–295 $m\mu$ with $E_{1\text{cm}}^{1\%}$ 346–360, and further chromatography has failed to raise the intensity of absorption. The preparations were practically free from glycerides or vitamin A. They underwent saponification with difficulty, but finally yielded approximately half their weight as recovered fatty acids after extracting the acidified soaps with ether. The unaponifiable material tended to decompose with consequent loss of absorption intensity, but in one case an $E_{1\text{cm}}^{1\%}$ (286 $m\mu$) value of 620 was obtained when quinol was used as antioxidant during the saponification. Kitol, which has been reported as possessing an $E_{1\text{cm}}^{1\%}$ value of 700 when obtained as freshly recrystallized material, is not very stable, even in the solid state in sealed ampoules, it does not withstand chromatography without decomposition and it is unstable towards alkali. If it is re-esterified, the esters are sometimes recrystallizable, but *cis-trans* isomerism may bring in complications.

The 'purified' kitol preparations of $E_{1\text{cm}}^{1\%}$ 350 are clearly mixed diesters containing an assortment of higher fatty acids of molecular weights exceeding 280. The preparation of such kitol esters appears to rule out the possibility that kitol is an artifact, and to show that kitol esters are present in whale liver. If the samples of blue- and sperm whale livers were representative, kitol esters are quantitatively much more significant in sperm whales than in blue whales. Too little, however, is known about variations between individual whales of either species to permit this observation to be generalized.

The fact that kitol is a di(vitamin A) is fully established, and from its absorption spectrum it must have a chain (or two chains) of four conjugated double bonds instead of the five conjugated double bonds of vitamin A. The molecular extinction coefficient of kitol is about 41,000 and that of vitamin A about 50,000.

Gridgeman, Savage & Gibson (1948), working at the Port Sunlight laboratories of Lever Bros and Unilever Ltd, have for some time been engaged upon the problem of determining vitamin A in whale liver oils. They have worked out an excellent method for the separation of vitamin A alcohol from unaponifiable extracts by chromatography, and we are indebted to Messrs Lever Bros for information and advice over a long period.

Comparing the method we have described with the procedure based on preliminary saponification it seems likely (a) that for oils in which much of the vitamin A is free there are advantages to be gained from completing the saponification in aiming at an estimate of the vitamin A content at the time of the test, (b) that for oils in which most of the vitamin A is esterified, direct chromatography is preferable in minimizing losses and in speed of operation, (c) that a determination of free and esterified vitamin A gives some indication of the probable stability of the oil, as it is the free vitamin A which forms anhydro vitamin A and probably some artifacts.

SUMMARY

1 The acetone soluble lipid from a sample of frozen blue-whale liver contained vitamin A ($E_{1\text{cm}}^{1\%}$ (328 $m\mu$) 36.7) with relatively little kitol. A similar extract from frozen sperm whale liver was richer in vitamin A and kitol (16 and approx 20%, respectively, as esters).

2 The fractionation of whale liver oils has been attempted in various ways. Free vitamin A is extracted by means of 50% aqueous ethanol, and vitamin A esters are separable from kitol esters by means of 80% aqueous ethanol.

3 Chromatography of whale liver oil on alumina (weakened by addition of 10% (w/w) water) effects a clean separation of vitamin A (esterified) from kitol (esterified). Kitol ester fractions showing $E_{1\text{cm}}^{1\%}$ approx 350 and approx 170 at 286 $m\mu$ were regularly obtained.

4 Kitol esters are difficult to saponify, and the free kitol tends to be decomposed unless precautions are taken. They contain, however, about half their weight of kitol ($E_{1\text{cm}}^{1\%}$ (286 $m\mu$) values of about 620 have been obtained for free kitol).

5 Kitol esters fed to rats are neither converted to vitamin A nor stored in the liver.

6 A chromatographic method for the determination of vitamin A in unaponified whale liver oils has been described and tested.

7 A fairly good separation of kitol from vitamin A in whale-liver unaponifiable fractions can be effected by extracting the vitamin A with 50% aqueous ethanol, but the volume used limits the method to analytical work.

8 Preliminary work on whale liver phospholipins suggests that kitol is not present in the true acetone insoluble lipid, but that it may occur in more than one type of combination

9 Vitamin A and possibly kitol is present in fin-whale milk

A small sample of kitol was kindly placed at our disposal by Dr Baxter of Distillation Products Inc. We are indebted to the Ministry of Food and the Medical Research Council for grants in aid of the work. In obtaining material we have had most friendly help from the whaling industry and many scientists engaged in it both in this country and abroad

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Studies in Vitamin A

13 THE ALLEGED FORMATION OF VITAMIN A FROM β CAROTENE TREATED WITH IODINATED CASEIN

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(Received 19 April 1949)

Kaplansky & Balaba (1946) claimed that β carotene in colloidal solution when incubated with either thyroglobulin or iodinated casein was converted into vitamin A in good yield, a typical experiment resulted apparently in the production of 60 μ g of vitamin A from 150 μ g of β carotene. If it be true that thyro active compounds can effect such a conversion, the finding is of major importance to the biochemistry of carotene and vitamin A. An investigation of Kaplansky & Balaba's claim was therefore undertaken. In the first part of the work the experimental procedure of Kaplansky & Balaba was followed as closely as possible, but when efforts to produce vitamin A failed, the conditions were then varied. A short report of the results of this work has already appeared (Cama & Goodwin, 1948).

EXPERIMENTAL

Preparation of iodinated casein. NaHCO_3 (3.6 g) was added to skimmed milk (500 ml.) freed from fat by repeated centrifugation, and the liquid heated to 40°. Small portions of I_2 were added with constant stirring until a slight excess remained at the bottom of the beaker (about 8.0 g of I_2 were required). The temperature was then raised to 65° for 24 hr., the liquid being constantly stirred. After cooling, the intensely yellow solution was filtered and the filtrate brought to pH 4.5 with acetic acid. The precipitated iodinated casein was washed, dissolved in very dilute alkali and reprecipitated with acetic acid. This process was repeated three times. The I_2 content of the product, determined according to the

micro method of Groák (1926) was 8.45–8.67%. Kaplansky & Balaba (1946) report 0.85%, but this appears to be a mistake.

Preparation of colloidal solutions of β -carotene. Crystalline β carotene (approx. 3 mg) was dissolved in a small volume of freshly redistilled diethyl ether (20 ml.) and 96% (v/v) ethanol added (5 ml.). The ether was removed by distillation on a water bath at 50–60°, the distillation was continued until a test portion of the distillate no longer separated into two layers after the addition of a small volume of water. The ethanol solution of β carotene was then diluted with distilled water and rapidly filtered, the colloidal solution was placed in a boiling water bath to remove the ethanol and any residual ether. The remaining concentrated colloidal solution was filtered again, diluted with water to approximately the desired concentration and used without delay.

Incubation of solutions. The details of Kaplansky & Balaba's (1946) method are as follows. To a known volume of colloidal solution containing 150 μ g β carotene is added iodinated casein (30 μ g) dissolved in weak alkali, the reaction mixture, after being adjusted to pH 7.3 with acetic acid, is incubated for 2 hr. at 37°, controls consisting of β carotene solutions only or β carotene solution + boiled iodinated casein adjusted to pH 7.3 are treated in the same way.

In the first stages of the present investigation the above procedure was followed as closely as possible. (Kaplansky & Balaba did not specify the concentration of the weak alkali or the acetic acid, we used 0.01N NaHCO_3 and 0.01N HOAc .) The procedure was later modified by altering (a) the pH (by the use of phosphate buffers), (b) the incubation times, and (c) the amounts of substrate and 'enzyme', and (d) by using desiccated thyroid or minced fresh thyroids instead of iodinated casein.

Determination of carotene and vitamin A Kaplansky & Balaba used the method of Rachevski for the determination of carotene, no reference or details were given, but a search of the literature indicates that the method used can only be that of Rachevski & Troitski (1938). It is not based on absorption spectrophotometry as are most methods of carotenoid analysis, but on iodometry. A known amount of iodine is added to a colloidal solution of carotene and the amount not absorbed is measured in the usual way by titrating with thiosulphate. Such a method, as will be noted later, has serious drawbacks when used in experiments of the type under discussion. We preferred the method in use in this laboratory, as it is unambiguous and well authenticated.

A measured volume of the colloidal solution is extracted three times with an equal volume of freshly redistilled diethyl ether, the ether extracts are combined and the solvent removed on the water bath, the residue is dissolved in a known volume of cyclohexane and the absorption spectrum recorded using a Beckman photoelectric spectrophotometer. Cyclohexane is chosen as the solvent so that vitamin A determinations, making use of both ultraviolet absorption and the SbCl_3 colour test, could be carried out on the same solution, this eliminates further manipulations involving heat, and minimal heat treatment is always to be aimed at when dealing with carotenoids.

Kaplansky & Balaba stated that they determined vitamin A by the Carr Price method (SbCl_3 reagent), and, in one experiment, by the spectrophotometric determination of the absorption maximum at $328 \text{ m}\mu$, but no details of the method were given. In the present investigation two procedures for determining vitamin A were used: (a) the Morton & Stubbs (1946) method of direct ultraviolet spectrophotometry, and (b) the SbCl_3 colour test. The Morton & Stubbs method is a well authenticated method in which allowance can be made for absorption not due to vitamin A. The SbCl_3 colour test was carried out using a Hilger Nutting visual spectrophotometer, this instrument allows the differentiation of the SbCl_3 vitamin A absorption maximum at $617 \text{ m}\mu$ from SbCl_3 carotene absorption maximum at $590 \text{ m}\mu$, even when carotene occurs in considerable excess over vitamin A.

RESULTS

Thirty six experiments were carried out under varying conditions, and in no case was the formation of vitamin A unequivocally demonstrated after incubation of β carotene with either iodinated casein, desiccated thyroid, or fresh thyroid brew, in one case only the Morton & Stubbs (1946) correction procedure pointed to the presence of a trace of vitamin A, but this was not confirmed by the antimony trichloride colour test.

In the first series of experiments Kaplansky & Balaba's (1946) conditions were reproduced as closely as the published directions permitted, later the conditions were varied and Table 1 indicates the limits of variation.

In Fig. 1 are shown the full absorption curves obtained in three typical experiments in which β carotene was incubated (a) alone, (b) with iodinated casein, and (c) with desiccated thyroid. It will be noted that although the curves do not

coincide, owing to the difficulty of obtaining colloidal carotene solutions of the same concentration (see p. 319), they are almost identical in shape, thus no differential changes in the β carotene have occurred.

Table 1 *Variations in the experimental conditions used in attempting to convert β -carotene into vitamin A by incubating with iodinated casein or desiccated thyroid*

Variable	Limits of variation
β Carotene	2.5–30 μg
Incubation time	2–2.5 hr
pH	4–8
Iodinated casein	50–60 μg
Desiccated thyroid	30–60 μg
Temperature	Not altered (37°)

by incubation under different conditions. The appearance of a small band at $336 \text{ m}\mu$ is due to *trans-cis* isomerization of the carotenoid produced under the action of heat, the $336 \text{ m}\mu$ band is Zechmeister's (1944) *cis* peak.

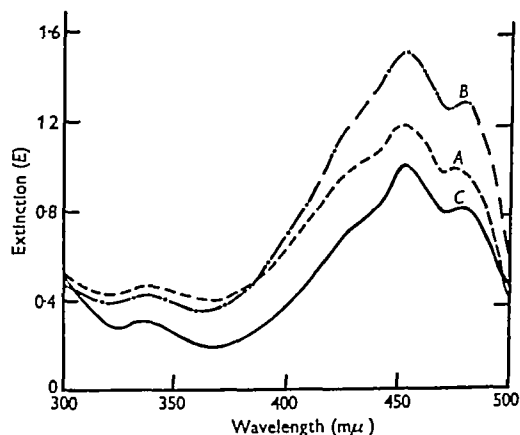


Fig. 1 The absorption spectrum of β carotene: A, after incubation alone for 2.5 hr at 37° ; B, after incubation with iodinated casein for 2.5 hr at 37° ; C, after incubation with desiccated thyroid for 2.5 hr at 37° .

DISCUSSION

It will be seen that we have not, in this laboratory, been able to detect the formation of vitamin A when colloidal solutions of β carotene are incubated with iodinated casein or desiccated thyroid. The apparatus used is known to be capable of detecting small amounts of vitamin A in the presence of carotene so there is no doubt that vitamin A was not present, it was not a matter of failure to detect it.

Kaplansky & Balaba (1946), on the other hand, report three experiments in which the yield of vitamin A was 45%. We have thus to attempt a reconciliation between contradictory experiences. It may be that favourable conditions are elusive, and

that although we tried to follow Kaplansky & Balaba's methods exactly we failed to achieve such conditions, it seems, however, that a method which produces a 45 % yield of vitamin A in the hands of one set of workers should in another laboratory at least yield detectable amounts. It is interesting to note that Wiese Lowry (1949) has informed us that she too was unable to repeat Kaplansky & Balaba's work. It is possible to criticize Kaplansky & Balaba's work from a technical viewpoint, and it is then clear that their claims are not securely based. Their technique may be considered under three heads.

(i) *Estimation of β carotene* The iodometric method used by Kaplansky & Balaba is at best unspecific, and it is difficult to understand why it was used if an optical instrument were available (as apparently there was, because in one experiment $E_{328\text{ m}\mu}$ was measured). The iodometric method presents a problem which Kaplansky & Balaba have not discussed. For example, the results of a typical experiment recorded by them indicates that 150 μg of β carotene after incubation with iodinated casein yielded 86 μg of β carotene and 55 μg of vitamin A. Now, vitamin A is an unsaturated compound and will itself absorb iodine, thus, unless this is allowed for, the iodometrically determined β carotene content of a mixture of β carotene and vitamin A will be incorrect. Assuming this correction has not been made, and there is no internal evidence that it has, the results just quoted are open to two serious objections. (a) If the β carotene not converted into vitamin A is reasonably stable to incubation, as they claim it is in the case of boiled iodinated casein, and as we find it is in all cases, then the iodometric method should give a value for ' β carotene' still in the neighbourhood of 150 μg . (b) If the value 86 μg was the determined uncorrected value then this must represent ' β carotene and vitamin A', and as 55 μg of vitamin A are reported only about 30 μg of β carotene have been accounted for out of an expected remainder of about 100 μg .

(ii) *Colloidal carotene solutions* Although following precisely the directions given by Kaplansky & Balaba, we found the production of the colloidal solutions to be much less reproducible than they did. For example, according to their table they used for each experiment exactly 150 μg of carotene obtained by diluting a concentrated colloidal solution a known amount. We could not make this method work, for, no matter how carefully the water was added to the concentrated carotene solution, a little was always thrown out of solution and had to be filtered off, thus it was impossible to obtain a solution of colloidal carotene of known strength merely by dilution, the content of each colloidal solution had to be measured each time.

(iii) *Measurement of vitamin A* Kaplansky & Balaba give no details of how they measured the

SbCl_3 colour test. How they differentiated between the carotene SbCl_3 colour test and the vitamin A SbCl_3 colour test, especially in a mixture of two, is not stated, so that it is impossible to assess the evidence.

Regarding the determination of vitamin A in the presence of excess of β carotene by ultraviolet spectrophotometry, β carotene itself absorbs in the 300–340 $\text{m}\mu$ region and thus has to be allowed for before the contribution of vitamin A to the absorption of a solution can be evaluated. This is even more important when β carotene solutions have been incubated, as increased absorption appears in the 300–400 $\text{m}\mu$ region owing to the formation of *cis* isomers from all *trans* β carotene. When a photoelectric spectrophotometer is available the correction procedure of Morton & Stubbs (1946) allows the determination of the vitamin A contributed.

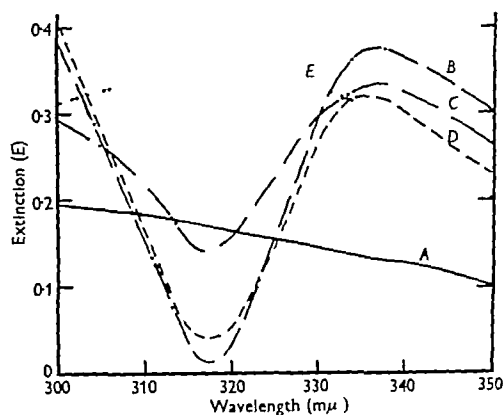


Fig. 2 The absorption spectra of β carotene and mixtures of β carotene and vitamin A in the spectral region 300–350 $\text{m}\mu$. A, pure β carotene, B, β carotene after incubation at 37° for 2.5 hr, C, β carotene after incubation at 37° for 2.5 hr with iodinated casein, D, a computed curve for a mixture of vitamin A and incubated β carotene, containing about 30 % of vitamin A, E, pure vitamin A.

The appearance of a carotene *cis* peak (Zechmeister, 1944) in the region of 330–335 $\text{m}\mu$ after incubation could easily be mistaken for vitamin A absorption, especially when a spectroscopy with a low dispersion is used. To illustrate this point a number of absorption curves are recorded in Fig. 2. The following points will be noted: (a) the reality of the *cis* peak of incubated carotene, (b) the difference between the curve of isomerized β carotene and that of a mixture of this β carotene and vitamin A (approx. 30 % vitamin A) is slight but well marked, and the presence of absorption due to the vitamin A in an incubated β carotene solution could be detected even without application of the Morton & Stubbs (1946) correction if the curve were measured

photoelectrically, (c) that this difference might be missed using old (photographic) methods, for the changes, involving a shift of the band maximum only 1–2 $m\mu$ and a small decrease in the slope on the short wave side of the maximum, are nearing the limits of detection, (d) that the curve of the β carotene incubated alone is little different qualitatively from that obtained after incubation with iodinated casein

Although experiments using more refined techniques have failed to substantiate earlier claims that thyroactive compounds catalyse the *in vitro* conversion of β carotene into vitamin A, it is not to be assumed that the thyroid has no action on the *in vivo* metabolism and vitamin A. In fact, it has recently been shown in this laboratory that desiccated thyroid

enhances and thiouracil reduces the absorption of β carotene from the intestinal tract (Cama & Goodwin, 1949)

SUMMARY

1 It has not been possible to substantiate the claim that iodinated casein and desiccated thyroid are active in converting β carotene into vitamin A *in vitro*

2 The most likely error leading to this conclusion is the failure to distinguish the *cis* peak of isomerized β carotene from the 326 $m\mu$ absorption band of vitamin A

We thank the Medical Research Council and the Ministry of Food for financial assistance, Prof. R. A. Morton for his interest and help, and Mr J. W. Lord (Messrs J. Bibby and Sons Ltd.) for the Russian translations

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The Pyruvate-oxidase System in Brain and the Tricarboxylic Acid Cycle*

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(Received 11 April 1949)

The term pyruvate oxidase system has been used in this laboratory to denote the complex of enzymes in brain which induce oxidation of added pyruvate. In earlier work (Long, Ochoa & Peters, 1939; Banga, Ochoa & Peters, 1939) the idea that this system in pigeon brain involved a tricarboxylic acid cycle (Krebs & Johnson, 1937; Krebs & Eggleston, 1940) was not supported. One of the main reasons was that, in contrast with kidney preparations, the increased oxygen uptake observed on addition of fumarate as well as pyruvate was not observed with citrate, which could not therefore be an intermediate, but, since then, the role of citrate has been changed to that of a side product rather than that of an

intermediate (Wood, Werkman, Hemingway & Nier, 1941). It has always been difficult to account for the action of fumarate upon any other view than that of a tricarboxylic acid cycle, and we now think that the pyruvate oxidase system in our homogenates does in fact include much of this cycle when fumarate is present, we are giving our evidence for this, though all details are not yet clear. The most important new point is that citrate and a substance, which is estimated as α -ketoglutarate, accumulate during the oxidation of pyruvate in the presence of fumarate and when malonate is also present.

METHODS

Estimations

Pyruvic acid. The Neuberg Case method (Case, 1932) has been used as modified by Peters & Thompson (1934), the final colour of the hydrazone in ethanolic KOH being

* A preliminary account was given to the Biochemical Society on 22 January 1949 (Coxon, Liébecq & Peters, 1949).

† With the technical assistance of R. W. Wakelin.

measured in a Hilger absorptiometer with filter no 7, the modified procedure described by Long (1942) has also been used. It has, however, been realized during this work that these methods give misleading results since they do not distinguish between pyruvate and α ketoglutarate in a mixture, though the extinction of the latter is less for a given concentration

The Friedemann & Haugen (1943) and the Straub (1936) methods have been employed as described by them, with the exception that in the case of the former a Beckman spectro photometer was used for the final reading as explained below in connexion with α ketoglutarate

α Ketoglutarate has been estimated most often by the method of Friedemann & Haugen (1943), employing the 420 and the 540 m μ . bands on the Beckman spectrophotometer instead of the simple photometer with different filters recommended by them. The enzymic method of Krebs (1938) was also used on a few occasions (as an independent check on the identity of the α ketoglutarate). It is conceivable, however, as will be discussed later, that the substance which was determined by either method as α ketoglutarate was originally present in the tissue suspension as oxalosuccinate and was converted to α ketoglutarate by the subsequent analytical operations. Provided that the mixture contains more than 10% of α ketoglutarate, the acids can be determined individually by the Friedemann & Haugen technique to an accuracy of $\pm 5\%$ in known mixtures. Duplicates agree to within $\pm 2\%$.

Citric acid has been determined by the usual colorimetric method of Pucher, Sherman & Vickery (1936) and acetic acid by Long's (1938) modification of the method of Weil Malherbe (1937). The details are given by Liébecq & Peters (1949). Latterly, preparatory to the citric acid estimation, all samples were boiled in presence of acid, the oxidation was carried out with permanganate, added drop by drop, and at 0.5N instead of 1.5N.

Preparations

Homogenates ('dispersions') from pigeon brain were made by grinding in a mortar according to the description of Banga *et al* (1939), they were either dialysed as described by these authors or, more recently, some of the homogenates have been centrifuged for 30 min in the cold room at 4000g, the centrifuged solid particles being then stirred into 1% KCl

solution after separation from the supernatant fluid, to give a 'centrifuged homogenate'. It should be emphasized that the most satisfactory 'ground' homogenates are made by adding the cold KCl solution to the sticky pounded brain tissue drop by drop in the initial stages. Weights quoted in tables are of fresh tissue.

Adenosinetriphosphate (ATP) was prepared from the Ba salt obtained commercially (Boots Pure Drug Co. Ltd.) and was 80% pure.

The following acids were prepared by Mr R. W. Wakelin in this laboratory: α ketoglutaric acid, m.p. 114.5 (corr.), *cis* aconitic acid from *cis* aconitic anhydride, m.p. 77°, *iso*-citric acid by hydrolysis of *iso*-citric acid lactone, m.p. 138° after several recrystallizations from ethyl acetate, this last m.p. is lower than that given by Krebs & Eggleston (1944), but the compound behaved normally with aconitase.

RESULTS

It is convenient to give first the new facts and then to consider these in relation to previous work.

Accumulation of citrate

The experiments given in Table 1 are representative of several, and show that with dialysed

Table 1 *Citrate formed in absence and presence of fumarate by dialysed homogenate of pigeon brain*

(Period of incubation 30 min, following equilibration for 10 min. Temp 38°. Tissue, approx 200 mg, fumarate, 0 or 10 μ mol, pyruvate, 30 μ mol, ATP, 1 μ mol, $MgCl_2$, 4 μ mol in 2.5 ml in each bottle with 0.08M phosphate buffer (pH 7.3). Addition of fumarate without pyruvate in control experiments produced somewhat less citrate than did pyruvate without fumarate.)

Exp no	Citrate formed (μ mol./bottle)		Difference due to fumarate
	Without fumarate	With fumarate	
1	0.36	2.43	+2.07
2	0.71	2.31	+1.60
3*	0.05	2.70	+2.65

* Centrifuged

Table 2 *Effect of cis aconitate and citrate upon oxygen uptake of centrifuged and dialysed brain dispersions*

(Tissue approx 200 mg/bottle, temp 38°. Period of exp 30 min. Each bottle contained 0 or 30 μ mol. pyruvate, 0 or 10 μ mol. fumarate, 1 μ mol ATP, 4 μ mol $MgCl_2$ and 0.08M phosphate buffer pH 7.3 in 2.5 ml.)

		Oxygen uptake ($\mu\text{mol/bottle}$)										
		Fumarate absent							Fumarate present			
Exp no	Concentration ($\mu\text{mol./bottle}$)		No other addi tions	<i>cis</i> Aconitate	Citra te	Pyr u vate	Pyr u vate + <i>cis</i> aconi tate	Pyr u vate +	No other addi tions	Pyr u vate	Pyr u vate + <i>cis</i> aconi tate	Pyr u vate + citra te + <i>cis</i> aconi tate
	<i>cis</i> Aconitate	Citra te										
1	45	0	—	—	—	5.0	6.7	—	2.5	9.8	—	—
2	40	9.6	—	—	—	—	—	—	3.0	12.8	13.0	10.0
3	15	11	—	—	—	—	—	—	—	12.7	—	12.5
4	20	20	2.8	2.5	2.3	4.6	5.6	4.5	3.9	7.3	—	—
5*	10	0	—	1.4	—	4.5	7.7	—	—	—	—	—
6*	10	0	—	2.3	—	4.2	8.0	—	—	8.9	—	—

Table 3 α Ketoglutarate estimated as formed in absence and presence of fumarate in dialysed homogenates of pigeon brain

(Tissue approx 200 mg /bottle, temp 38° Each bottle contained ATP, 1 μ mol, $MgCl_2$, 4 μ mol with 0.08M phosphate buffer at pH 7.3, 0 or 10 μ mol fumarate, and pyruvate as indicated, in 3 ml)

Exp no	Pyruvate added (μ mol)	Period of incubation (min)	α Ketoglutarate found (μ mol./bottle)		
			Fumarate absent	Fumarate present	Method of estimation
1	30	40	1	3.7	Friedemann & Haugen (1943)
2	18	40	Nil	2	
3	9	40	1	2.8	
4	30	10	Nil	2.5	
		40	Nil	3.5	
5	9	10	0.5	1.5	
		40	1	2.8	Krebs (1938)
6	9	40	2.4	4.5	
7	9	40	Nil	5.0	
8	0	40	Nil	0.35	Friedemann & Haugen (1943)
9	0	40	Nil	0.25	

brain homogenates respiring in pyruvate, citrate is formed, and that upon addition of fumarate much more citrate accumulates than in its absence. It was shown previously (Banga *et al* 1939) that addition of citrate does not produce a 'fumarate' effect in these homogenates, hence any two carbon fragment from pyruvate, after condensation with oxaloacetate and formation of citrate via *cis* aconitate, cannot be oxidized further. Addition of *cis* aconitate, together with pyruvate, gives some extra oxygen uptake in the absence of fumarate, tending to be less than that produced by fumarate, with fumarate and pyruvate there is no further increase. Citrate is inhibitory under some conditions (Table 2). Of other intermediates in the cycle, α -ketoglutarate is known from previous work in this laboratory to be oxidized by the brain preparation (see p 323). The simplest interpretation of these facts is that in the brain homogenates some form of tricarboxylic acid cycle is operating, and that the oxidation sequence tends to be choked between *cis* aconitate and α ketoglutarate so leading to the accumulation of citrate.

Formation of α ketoglutarate

During the respiration period of a brain homogenate, the concentration of a substance estimated as α ketoglutarate is greatly increased if fumarate is present (Table 3). In the presence of pyruvate this can be demonstrated only by Friedemann's spectrophotometric method in the mixed 2,4-dinitrophenylhydrazones in alkaline solution. It cannot be demonstrated with the various modifications of the Neuberg Case method (Case, 1932), though its presence can be deduced by estimating the total hydrazone in this way and subtracting the value for pyruvate obtained by the more specific

procedure of Straub (1936). The enzymic method of Krebs (1938) provides an independent means of assay.

Acetic acid

The formation of acetate ion has not been so thoroughly investigated, but Table 4 shows that its behaviour in presence and absence of fumarate is the converse of that occurring with citrate and α ketoglutarate. This indicates that, in absence of fumarate, the pyruvate is largely degraded to acetate, is consistent with the hypothesis advanced by Long & Peters (1939).

Table 4 Formation of acetate in absence and presence of fumarate by dialysed homogenate of pigeon brain

(Each bottle contained ATP, 1 μ mol, $MgCl_2$, 4 μ mol, 0.08M phosphate buffer (pH 7.3), pyruvate, 30 μ mol, either 0 or 10 μ mol fumarate and tissue, approx 200 mg in 2.5 ml. Period of experiment 30 min + 10 min equilibration)

Exp no	Acetate found (μ mol /bottle)	
	Fumarate absent	Fumarate present
1	—	3.16
2	—	2.70
3	13.25	4.42
4	10.9	—

The effect of malonate and the origin of α ketoglutarate

One of the important facts used in the proof of a tricarboxylic acid cycle in other tissues has been that there is formation of succinate from fumarate in the presence of excess of malonate (Krebs & Eggleston, 1940). Since excess of malonate inhibits the action of succinic dehydrogenase, it should also stop any possible formation of α ketoglutarate from fumarate via succinate. That the α ketoglutarate

Table 5 *Effect of malonate on oxygen uptake, disappearance of pyruvate and formation of citrate and α ketoglutarate*

(Each vessel contained ATP, 1 μ mol, $MgCl_2$, 4 μ mol, 0.08M phosphate buffer (pH 7.3), 10 μ mol pyruvate and dialysed brain homogenate equivalent to 150 mg tissue. Malonate (where added), 0.025M. Time of incubation was 30 min + 10 min equilibration. Temp 38°. All figures given are in μ mol/bottle, but the O_2 values have not been corrected for the equilibration period.)

	Exp 1				Exp 2		
	Pyruvate alone	With fumarate (10 μ mol)	With malonate	With fumarate + malonate	Alone (10 μ mol)	With fumarate + malonate	With fumarate + malonate
Pyruvate disappearance	3.5	8.1	3.0	6.7	5.5	7.5	6.5
O_2 uptake	2.2	6.9	1.4	5.0	1.6	6.7	4.7
Citrate formation	—	2.5	0.15	2.4	—	—	—
α Ketoglutarate formation	0.37	2.7	0.6	2.3	0.20	1.5	1.2

found in our experiments cannot be formed in this way is shown by Table 5, citrate and α ketoglutarate are produced in presence of malonate.

It seems certain that the substance estimated in this work is α ketoglutarate. 2,4-dinitrophenyl hydrazone. Not only does it behave spectrophotometrically in the same way as an authentic specimen, but also the substance can be converted into succinate and estimated enzymically as such. Nevertheless, there are some facts which make it uncertain at first sight whether this substance is present in the bottles as α ketoglutarate, its amount is not appreciably increased by isocitrate (Table 6) and in

unstable that it would not long remain unchanged. The experiments with comparatively low concentrations of α ketoglutarate given in Table 7 show

Table 7 *Fate of α ketoglutarate added in vitro*

(Each bottle contained 1 μ mol ATP, 4 μ mol $MgCl_2$, 0.08M phosphate buffer at pH 7.3, together with dialysed brain dispersion equivalent to 200 mg tissue. Volume/bottle 2.5 ml. Preliminary equilibration for 10 min. Temp 38°.)

Exp no	Malonate added (μ mol)	α Ketoglutarate (μ mol)		
		Initial	After 10 min	After 20 min
1	0	3.0	—	0.8
	0	6.0	—	1.7
	7.0	6.0	—	2.2
2	0	3.0	1.5	1.1
	0	6.0	2.9	2.1
	7.0	3.0	1.5	1.4

Table 6 *Comparison of α ketoglutarate formation in presence of cis-aconitate and isocitrate with that in presence of fumarate*

(Each bottle contained 10 μ mol pyruvate, 1.0 μ mol ATP, 4.0 μ mol $MgCl_2$ and 0.08M phosphate buffer at pH 7.3. Final volume 3 ml./bottle. Temp 38°.)

Exp no	cis Aconitate added (μ mol)	isocitrate added (racemic) (μ mol)	Fumarate added (μ mol)	α Keto glutarate found (μ mol)
1	10	0	0	0.75
	0	0	10	3.75
2	10	0	0	0.5
	0	0	10	2.5
3	0	20	0	0.1
	0	0	10	2.0
4	0	20	0	0.3
	0	0	10	2.0
5*	0	20	0	0.6
	0	0	10	1.8

* $MnSO_4$, 4 μ mol and coenzyme II, approx 1 μ mol, also added.

presence of malonate no extra α ketoglutarate appears, α ketoglutarate itself is known to be oxidized by the brain tissue. In view of this, we have considered the possibility that the ' α keto glutarate' is originally present as oxalosuccinate (Ochoa, 1948), however, both the statements of Ochoa and our observations suggest that this is so

that, though larger amounts are readily oxidized, smaller amounts tend to remain as such and their rate of disappearance is not greatly influenced by malonate. Hence the behaviour resembles that in our experiments, and indicates that the α keto glutarate found can be simply that accumulating in the system during the oxidation.

Oxygen uptake and the balance sheet

In the past, it has been thought possible to draw up a balance sheet indicating that 1.5 mol oxygen was used per mol pyruvate (Banga *et al.* 1939), whilst the oxygen:pyruvate ratio for the extra metabolism produced by adenylic acid in the presence of fumarate has been found to approach more nearly the theoretical ratio for complete oxidation, viz. 2.5 (Long, 1946). In absence of adenylic acid or fumarate the ratio is considerably lower. Such calculations have been based on the assumption that the action of fumarate was a simple catalysis. Since we now consider that the fumarate enters into a complex series of oxidations such a balance sheet is unsatisfactory, less oxygen is used than would be necessary for complete oxidation of pyruvate and

we are forced to believe that incompletely oxidized products are left at various stages in the cycle. No balance sheet can be made until full information is available of the amounts present of all partially oxidized products.

DISCUSSION

Our experiments have led us to the belief that, as shown in other tissues, fumarate increases the oxygen uptake with pyruvate in brain because it supplies oxaloacetate, and that therefore the total pyruvate oxidase system in brain, as understood in this laboratory, includes some form of 'tricarboxylic acid' cycle. Though there are still discrepancies to be explained, these do not justify any longer a rejection of the idea of oxidation via a cycle, which is consistent with so much other evidence. At the same time, it is not yet clear just how citrate is formed and there are other incongruities which should be mentioned. Unlike kidney preparations, the brain homogenates do not oxidize citrate, they oxidize *cis* aconitate only to a small extent. Further, as mentioned, added *cis* aconitate gave rise to much smaller amounts of ' α ketoglutarate' than did fumarate in a few experiments made by us (Table 6). We do not feel that the latter fact necessarily means that the α -ketoglutarate formed was arising by a separate path, because the conditions may be different when *cis* aconitate is formed as an intermediate in presence of plentiful fumarate. The simplest view is that in the preparations used here the cycle is partially blocked between the stages *cis* aconitate and α ketoglutarate and also between α ketoglutarate and succinate, but this interpretation still lacks the decisive proof that addition of some factor can remove the block.

Considering the oxidation in presence of fumarate, Banga *et al* (1939) found that both adenine nucleotides and fumarate (as well as other acids in this series) were involved in the more complete oxidation of pyruvate. In further careful work, Long (1946) concluded that the same amount of pyruvate disappeared in presence and absence of fumarate, but that with fumarate, there was a more complete oxidation of the pyruvate disappearing. This led him to consider that the idea advanced by Banga *et al* (1939), that fumarate increased the utilization of pyruvate, was erroneous, no allowance having been made for pyruvate formed during the experiment by oxidation of fumarate to pyruvate. It is shown here that part of the α -keto acid estimated by

Neuberg Case methods with fumarate present is α ketoglutarate, which interferes only to an exceedingly small extent in the Straub (1936) method used by Banga *et al* (1939). Hence the discrepancy might be explained by the failure of specificity in the Neuberg Case methods. Nevertheless, the idea that the function of the fumarate is not simply catalytic is correct. Doubtless an exact knowledge of the amounts of various components of the cycle left in a partially oxidized state would lead to a quantitative balance sheet in these brain preparations, but, since so much will depend upon uncertain factors in the fragmentation, this investigation seems of secondary importance at the present time when various features of the cycle are more readily studied in other tissues (cf Green, Loomis & Auerbach, 1948).

In absence of fumarate, the oxidation of pyruvate takes a different course and seems to go to acetate rather than *cis* aconitate, as mentioned, this is consistent with the hypothesis of Long & Peters (1939), with the observations of Banga *et al* (1939) in absence of fumarate, and with later work by Long (1943).

It is hoped to deal with this reaction later, and to leave to a later communication also the explanation of the apparent discrepancy between these results and some earlier reports from this laboratory based upon the assumption that succinic dehydrogenase and the pyruvate oxidase system were acting independently in brain. Some of the implications of these experiments for pharmacology have been discussed elsewhere (Peters, 1948).

SUMMARY

1 An accumulation of citrate and of α ketoglutarate has been found to accompany the oxidation of pyruvate by dialysed 'dispersions' (i.e. finely ground homogenates) of pigeon brain in a medium containing fumarate and reinforced with magnesium ions and adenine nucleotide.

2 This evidence, despite the previous observation in this laboratory that citrate cannot replace fumarate in its effect on the oxygen uptake of these preparations, is taken to favour the operation in them of a tricarboxylic acid cycle when fumarate is present.

3 When fumarate is absent, the oxidation of pyruvate is associated with the formation of acetate.

We are grateful to Miss M. Pollock for help in some of these experiments.

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Acceleration of Bacterial Glutamic Decarboxylase and Glutaminase by Cetyltrimethylammonium Bromide

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(Received 11 April 1949)

It has been shown in a previous paper from this laboratory (Krebs, 1948) that cetyltrimethyl ammonium bromide ('cetavlon') can accelerate the decarboxylation of glutamate and the deamidation of glutamine by washed suspensions of *Clostridium welchii*. The present paper is concerned with the mechanism of this effect. As it is known that detergents may alter the permeability of cells (Gale & Taylor, 1947; Hotchkiss, 1946), it was thought that cetavlon might facilitate the entry of the substrates into the cells. To test this hypothesis, the effect of cetavlon on the rate of decarboxylation of glutamate and glutamine by cell free extracts has been examined, but contrary to expectation the detergent gave the same effect as with intact cells. Thus the effect cannot be due to changes in the permeability of the cell wall.

METHODS

Organisms. *Clostridium welchii* S.R. 12 (National Collection of Type Cultures (N.C.T.C.) no. 6784) was grown for 16 hr. at 40° and a strain of *Escherichia coli* isolated locally and *Proteus morganii* (N.C.T.C. no. 2818) for 24 hr. at 30°, on the medium described by Krebs (1948). *Bacterium cadaveris* (N.C.T.C. no. 6788), *Cl. welchii*, (N.C.T.C. no. 6785), and *Streptococcus faecalis* (N.C.T.C. no. 6783), were grown as described by Gale (1947), but 'Pronutr' (a commercial casein hydrolysate marketed by Herts Pharmaceuticals Ltd., Welwyn Garden City) was used instead of tryptic casein

Clostridium septicum (N.C.T.C. no. 547) served as a source of ornithine decarboxylase. The activity of the ornithine decarboxylase was increased 25 fold by reinforcing Gale's (1947) medium with extra ornithine (1 mg./ml. medium).

After growth, the cultures were centrifuged, the cells washed twice with 0.9% NaCl, and suspended in the appropriate buffer (Table 6). Phosphate buffer was used for the pH range from 7.0 to 5.5, acetate buffer for the range 5.5 to 4.0 and lactate buffer for the pH range from 4.0 to 2.5. The final concentration of the buffer was 0.10M.

Chemicals. The preparations of L-glutamic acid, L-glutamine and cetavlon used in this work were those described by Krebs (1948). Ornithine was prepared from male herring roe by the following method. The roe (250 g.) was heated on the water bath in a mixture of 150 ml. of conc. HCl and 50 ml. of TiCl₄ (20%). During the heating the roe was broken up with a glass rod and when fairly uniform it was autoclaved at 120° for 45 min. Water and HCl were removed by distillation *in vacuo* on a steam bath. The residual gum was dissolved in 100 ml. of hot water and 40% NaOH added until the pH was 6. The solution was filtered and, after adjustment of the pH to 7.6, an arginase preparation (15 ml. of 10% homogenized fresh rat liver) was added, and the mixture was incubated overnight. Urea was estimated manometrically and the ornithine content calculated from the urea formed.

Chemical determinations. The decarboxylation of glutamate and of glutamine was followed by measuring the evolution of CO₂ in a Warburg manometric apparatus. Buffer and substrate solutions were usually placed in the main compartment of the cup and the cells or extracts (see p. 326) containing the bacterial decarboxylase in the side arm. The gas space

was filled with N_2 , and a stick of yellow phosphorus was put into the centre well to eliminate O_2 . After 10 min equilibration, the contents of the side arm and the main compartment were mixed. Readings were taken at 2 or 4 min. intervals. The rate of reaction is expressed as Q_{CO_2} , i.e. μl CO_2 /mg dry wt of cells/hr. In the case of extracts the Q_{CO_2} was calculated not from the dry wt of extract used, but from the dry wt of the cells from which the extract was made. The total volume of the fluid in each Warburg cup was 2.2 ml and the amount of enzyme material added was generally equivalent to 2.0–5.0 mg intact cells, varying with the activity of each batch of material, which was determined by a preliminary experiment. The concentration of enzyme material was such that not more than 50% of the added substrate was decomposed in the first 10 min, i.e. not more than 112 μl CO_2 was produced. Within a suitable range the rate of decarboxylation of glutamine in extracts was proportional to the enzyme concentration. Under the conditions used the range was between 1 and 20 mg/cup in the absence of cetavlon and 1 and 10 mg in its presence. At higher concentrations the rate was too rapid for precise measurement. At lower concentrations the rate fell off after about 5 min, possibly owing to the destruction of the enzyme in dilute solution. Under these conditions the rate was approximately linear for at least 10 min and the Q_{CO_2} was calculated from the linear rate of gas evolution. Any deviation from this procedure is mentioned later. Ammonia was determined by the method of Parnas & Heller (see Parnas, 1934).

Preparation of cell free extracts The washed cells were broken by grinding with powdered pyrex glass (Wiggert, Silverman, Utter & Werkman, 1940; McIlwain, Roper & Hughes, 1948). The degree of cell disintegration was checked by microscopic examination of a Gram stained smear of the ground mixture diluted with water. Usually no intact cells were found, and the cell debris of *Cl. welchii* was Gram negative. The clay like mass of broken cells and glass was mixed with 0.05 M acetate buffer, pH 4.6, in the proportion of 2 ml of buffer for 1 g of wet cells, and centrifuged for 10 min at about 4000 rev./min. The supernatant was poured off and the residue extracted twice more with the same amount of buffer. The three extracts were combined, centrifuged again, and the slightly opalescent solution stored at about 2° in a stoppered cylinder.

Extracts of *Cl. welchii* contained about 80% of the original glutamic decarboxylase activity (see Table 1). Extracts of equal potency were obtained when the above buffer was replaced by other solutions (0.9% NaCl, 0.05 M acetate buffer pH 5.0 or 6.0, or 0.05 M phosphate buffer pH 7.0). The yield of glutaminase was difficult to measure with any certainty owing to the change in behaviour of the enzyme after extraction. For instance, when the rate of decarboxylation of glutamine was measured at pH 4.9 the yield of glutaminase appeared to be 50–60% without cetavlon and 80–90% when it was added. When the activity was measured at pH 4.1 the yield of glutaminase appeared to be negligible in the absence of cetavlon, but when the detergent was added the activity was often greater than that of the intact cells (see Table 1).

Extracts of *Proteus morganii* decarboxylated both substrates more slowly than the intact cells both in the absence and presence of cetavlon.

Esch. coli gave extracts which under the conditions used appeared to contain 25–40% of the original decarboxylase and 50–100% of the original glutaminase activity.

Stability of the enzyme preparations As already reported (Krebs, 1948) the decarboxylase and glutaminase in intact

cells of *Cl. welchii* suspended in 0.05 M acetate buffer, pH 4.5, and stored at about 2° keep for several weeks. The glutamic decarboxylase activity of an extract from *Cl. welchii* showed no decrease after 9 days at 2°, but a decrease of 35% after 30 days at 2°. In the same sample, the ability to decarboxylate glutamine fell off more rapidly, 30% of the original activity was lost in 9 days and 55% after 30 days at 2°. In another experiment, in which an extract was incubated without substrate for 20 min at 40° in 0.1 M acetate buffer pH 4.6, 32% of the glutamic decarboxylase and 80% of the glutaminase activity disappeared. Thus the glutaminase in the extracts is less stable than the glutamic decarboxylase. Most of the work described in this paper was carried out with glutamic acid as the substrate in order to avoid complications arising from instability of the glutaminase.

RESULTS

The effects of cetavlon on the rate of decarboxylation of glutamate and glutamine in intact cells and extracts Table 1 shows that cetavlon accelerates the decarboxylation of glutamate and glutamine in extracts and in washed cells of *Cl. welchii*, *Proteus morganii* and *Esch. coli*.

Table 1 *The effect of cetavlon on the rate of decarboxylation of glutamate and glutamine by intact cells and extracts*

(Temp. 40°, substrate, 0.0045 M, cetavlon, 0.0025 M, 0.1 M acetate buffer pH 4.6)

Substrate added	Q_{CO_2}	
	Intact cells	Extract
<i>Cl. welchii</i>		
Glutamate	270	224
Glutamate and cetavlon	535	405
Glutamine	114	5
Glutamine and cetavlon	270	290
<i>Proteus morganii</i>		
Glutamate	40	44
Glutamate and cetavlon	310	82
Glutamine	45	23
Glutamine and cetavlon	250	56
<i>Esch. coli</i>		
Glutamate	102	11
Glutamate and cetavlon	425	160
Glutamine	89	40
Glutamine and cetavlon	270	270

With intact cells of *Cl. welchii* at pH 4.1 the degree of acceleration of the decarboxylation of glutamate on the addition of cetavlon was from two to three fold. In extracts the effect of cetavlon was slightly less than with the intact cells, but of the same order. On the other hand, the decarboxylation of glutamine showed striking differences between intact cells and extracts of *Cl. welchii* (see Table 1). In the absence of cetavlon the extracts were 20% less active than the intact cells even at pH 4.9, which has been shown to be the optimum pH for the decarboxylation of glutamine (Krebs, 1948). The low activity of the

extracts towards glutamine is not due to an irreversible destruction of the enzymes because the addition of cetavlon greatly increases the activity, in some cases to a Q_{CO_2} level higher than that of the intact cells

In the case of *Proteus morganii* the acceleration by cetavlon was more marked in intact cells than in extracts. It will be shown later for *Cl welchii* that the pH and the substrate concentration can greatly influence the effect of cetavlon. The role of these factors has not been investigated for *Proteus morganii*.

In *Esch coli* the effects of cetavlon were very pronounced both on intact cells and on extracts, irrespective of whether glutamate or glutamine was the substrate.

The effect of cetavlon on the pH curve of the glutamic decarboxylase in extracts of *Clostridium welchii*. As shown in Fig. 1 addition of the detergent to extracts

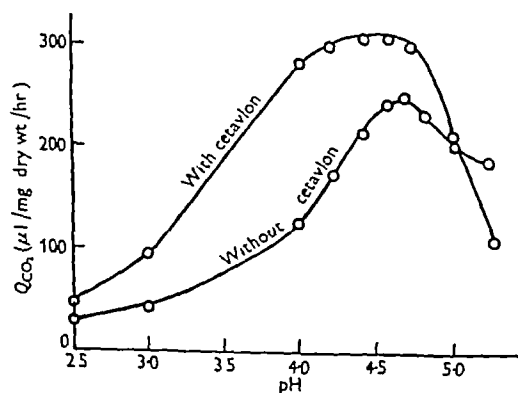


Fig. 1 The effect of cetavlon on the pH curve of the extracted decarboxylase of *Cl welchii*: 0.1 M lactate, pH 2.5–3.0, 0.1 M acetate, pH 4.0–5.0, 0.0025 M cetavlon if added, substrate 0.0045 M glutamate, extract corresponding to 2.0 mg dry wt of cells, total volume 2.2 ml, temp 40°

of glutamic decarboxylase from *Cl welchii* changed the shape of the pH curve of the enzyme by broadening the optimum towards the lower pH range. This effect is similar to that found with intact cells (Krebs, 1948). Above pH 4.8 inhibitions varying from 5 to 20% were found on the addition of cetavlon to both extracts and cells. As the degree of acceleration by cetavlon was greatest between pH 4.0 and 4.2 its effect has been measured at pH 4.1.

It is noteworthy that the pH optimum in the absence of cetavlon is 4.6 with extracts and 4.1 with cells. Similar changes in the optimum pH of the tyrosine decarboxylase on extraction from *Strep faecalis* have been described by Epps (1944). Another point of interest is that the extracted enzyme has a sharper pH optimum than has the enzyme of the intact cells.

The effect of cetavlon on the stability of glutamic decarboxylase in extracts of *Clostridium welchii*. There was loss of activity of the decarboxylase without the addition of cetavlon when the extract was incubated at 40° at pH 4.9, 4.1, 3.0 and 2.5 (Table 2). Cetavlon had little effect on this loss of

Table 2 The effects of cetavlon on the stability of the glutamic decarboxylase in extracts of *Clostridium welchii*

(Main compartment 1.0 ml 0.2 M buffer, 0.5 ml extract, equivalent to 2.0 mg dry wt cells, if added, 0.1 ml 2% cetavlon. Side arm 0.5 ml 0.01 M glutamate. Total volume 2.2 ml. Contents mixed after 10 min or 20 min at 40°)

Buffer	Q_{CO_2} at 10 min		Q_{CO_2} at 20 min	
	Without cetavlon	With cetavlon	Without cetavlon	With cetavlon
Acetate pH 4.9	264	264	180	180
Acetate pH 4.1	142	250	82	160
Lactate pH 3.0	52	100	26	124
Lactate pH 2.5	33	99	10	43

activity. The accelerating effect of cetavlon after partial destruction of the decarboxylase was about the same as before incubation. The detergent therefore neither destroys the activity of the enzyme nor protects it from destruction.

The effect of cetavlon on the solubility of the extracted decarboxylase of *Clostridium welchii*. On adjusting the pH of extracts to below pH 4.3 with acetate buffer a precipitate was formed. About 60% of the decarboxylase activity was precipitated at pH 4.1, and both the precipitate and the mother liquor gave the cetavlon effect (Table 3, Exp. 1). In the presence of cetavlon almost all the decarboxylase was in the precipitate (Table 3, Exp. 2), and the activity of the washed precipitate was not increased by the addition of cetavlon. At pH 4.6, the addition of cetavlon produced a precipitate, which, even after washing, contained decarboxylase whose activity was again unaffected by the addition of further cetavlon. About 70% of the activity was recovered in the precipitates formed when cetavlon was present, but quantitative assay was difficult as the precipitates were very sticky and adhered to pipettes and test tubes. The material was insoluble in acid and neutral buffers and lost activity if the pH was above 7.0. On the other hand, precipitates obtained by adjusting the pH of the extract to pH 4.1 without the addition of cetavlon redissolved at pH 6 and were stable even at pH 9. Above pH 9 the activity was lost rapidly.

If the extract was adjusted to pH 4.1 in the presence of methyl orange the precipitate was stained orange when cetavlon was absent, and this orange colour was removed slowly by washing. When cetavlon was present the precipitate was bright yellow and the colour was not removed after four

Table 3 *The effect of pH and cetavlon on the precipitation of the glutamic decarboxylase*

(Each fraction was transferred in 0.5 ml water to the side arm, 0.0045 M glutamate, 0.1 M acetate buffer pH 4.1 and cetavlon, if added, 0.0025 M, were in the main compartment. Total volume 2.2 ml.)

Condition of precipitate	Fraction tested	Q_{CO_2}	
		Without cetavlon	With cetavlon
Control Original extract not precipitated		108	344
Exp 1 Extract taken to pH 4.1 with acetate, left 15 min at 2°, centrifuged, precipitate washed once with 0.05 M acetate buffer pH 4.1	Supernatant	59	118
	Precipitate	88	188
Exp 2 Extract precipitated as above but in the presence of 2.5×10^{-3} M cetavlon, precipitate washed once with 0.005 M acetate buffer pH 4.1	Supernatant	0	0
	Precipitate	258	258
Exp 3 Extract adjusted to pH 4.6, diluted to the same volume as the above experiments and cetavlon added (final concentration 2.5×10^{-3} M)	Supernatant	0	0
Precipitate washed as above	Precipitate	262	262

washings with 0.05 M acetate buffer pH 4.6 or 4.1, or with 0.05 M phosphate buffer, pH 7.0. Hartley (1923) has shown that some cationic detergents change methyl orange to a bright yellow colour. This colour change is not due to any change in pH and is connected with micelle formation by the detergents. The fact that methyl orange becomes yellow when absorbed on to precipitated enzyme material indicates that detergent protein complexes have been formed (Putman & Neurath, 1945, Lundgreen, 1945).

Microscopic examination showed that the precipitate obtained by acidifying the extract to pH 4.1 was composed of very fine granules when cetavlon was absent, and coarse granules when the detergent was present. When these precipitates were shaken with glutamate at 40° the one without cetavlon did not change in appearance, whilst the one with cetavlon became coarser. This effect is similar to that found by Klarman & Wright (1946) on the clumping of bacteria by detergents. Thus cetavlon neither solubilizes nor peptizes the enzyme containing material, but precipitates it and forms complexes.

The effect of cetavlon on the rate of decarboxylation at varying concentrations of glutamate. At 30° the rate of decarboxylation of glutamate (2 mg dry cells in 2.2 ml, substrate concentrations 0.0023–0.046 M) was found to be steadier than at 40°, and the following experiments were therefore carried out at 30°. Table 4 shows the effect of cetavlon on the rate of decarboxylation at different glutamate concentrations by washed cells and extracts. It will be seen that the effect of the detergent was greatest at the lowest substrate concentrations, whilst at high substrate concentrations when the enzyme is almost saturated with substrate it has little or no effect. The increase being, in the case of the cells, 147% at 0.0023 M glutamate and only 13% at 0.046 M glutamate. These results can be explained on the assumption that cetavlon increases the apparent affinity of the substrate for the enzyme. The concentrations of substrate giving half the maximum velocity, determined graphically from the results

given in Table 4, are as follows: intact washed cells, without cetavlon, 0.009 M, with cetavlon, 0.004 M. Extract of cells, without cetavlon, 0.0095 M, with cetavlon, 0.0045 M.

Table 4 *Effect of cetavlon on the rate of decarboxylation at varying concentrations of glutamate*

(In each Warburg cup, 0.1 M acetate buffer (pH 4.1), glutamate solution, 0.0025 M cetavlon, if added, enzyme in the side arm, total volume 2.2 ml, temp 30°)

Glutamate (M)	Q_{CO_2}	
	Without cetavlon	With cetavlon
<i>Intact washed cells</i>		
0.046	890	1003
0.023	790	990
0.011	535	745
0.0046	268	465
0.0023	109	268
<i>Extract of the above cells</i>		
0.046	535	675
0.023	492	582
0.011	330	465
0.0046	211	362
0.0023	125	231

Assuming that the half maximum velocity is a measure of the apparent affinity of the enzyme for the substrate, the addition of cetavlon increased the affinity of the decarboxylase about twofold, both with intact cells and with extracts.

Changes in the apparent affinity of enzyme for substrate can be brought about by competitive inhibitors, addition of a competitive inhibitor decreasing, and its removal increasing, the apparent affinity. The effect of cetavlon could thus be explained by the assumption that it removes a competitive inhibitor which accompanies the bacterial decarboxylase and glutaminase. This conception is supported by the graph shown in Fig. 2, in which a plot is made of the reciprocal of the velocity $1/v$ against the reciprocal of the substrate concentration $1/S$ (Lineweaver & Burk, 1934). The two lines referring to extracts show the same ordinate inter-

cept, i.e. the same maximum velocity, but the addition of cetavlon has decreased the slope. According to Lineweaver & Burk (1934) this is expected if a competitive inhibitor is removed.

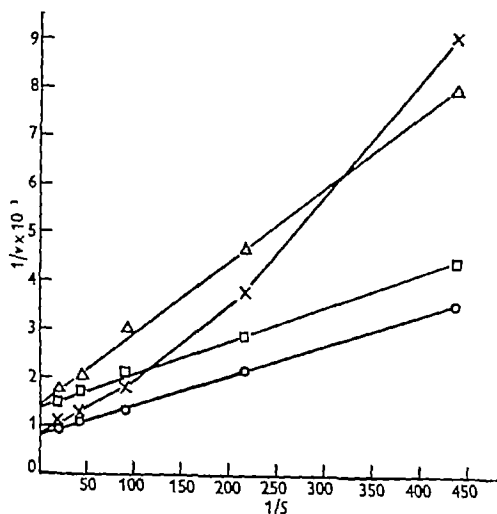


Fig. 2. Data in Table 4 plotted as suggested by Lineweaver & Burk (1934), v = reaction velocity, expressed as Q_{CO_2} , S = substrate concentration (M). \times — \times , intact cells without cetavlon, \circ — \circ , intact cells with cetavlon, Δ — Δ , extract without cetavlon, \square — \square , extract with cetavlon.

The plot of the data obtained with intact cells, when cetavlon is absent and the substrate concentration is low, does not give a straight line. This suggests that under these conditions the rate of entry of glutamate into the cell limits the rate of decarboxylation. This conclusion is further supported by the fact that a straight line is obtained from the data with extracts.

The plot obtained from the results with intact cells in the presence of cetavlon shows two points of interest. First, the line is straight, suggesting that cetavlon eliminates diffusion as a controlling factor. Secondly, the line is almost parallel to that showing the effect of cetavlon in the extract. This means that although cetavlon shows an effect on the rate of entry of glutamate into the cell, the apparent affinity of the enzyme in intact cells is also increased, in the same way as in extracts. The fact that the acceleration by cetavlon in intact cells of *Cl. welchii* is slightly higher than that found in extracts (see Table 1) can probably be explained by an additional effect of the detergent on the permeability of the cell.

The effect of cetavlon on the rate of decarboxylation of glutamine at varying substrate concentrations. The rates of decarboxylation at varying concentrations of glutamine by extracts of *Cl. welchii* are shown in Table 5. A concentration of substrate sufficient to saturate the enzyme was not reached, as the solu-

bility of glutamine at 30° is approximately $0.23M$. The decarboxylation is extremely slow at low concentrations of substrate without cetavlon and is increased by the addition of cetavlon, the cetavlon effect decreasing as the substrate concentration is raised. This suggests that the apparent affinity of the glutaminase is increased in a manner similar to that of the decarboxylase.

Table 5. *The effect of cetavlon on the rate of decarboxylation of varying concentrations of glutamine by extracts of Clostridium welchii*

($0.1M$ acetate buffer pH 4.1, glutamine and $0.0025M$ cetavlon in the centre, enzyme in the side arm, total volume $2.2ml$, temp 40°)

Glutamine (M)	Q_{CO_2}	
	With cetavlon	Without cetavlon
0.23	1160	440
0.12	850	250
0.046	490	143
0.023	330	78
0.0124	260	24
0.0046	215	10.5
0.0023	144	10.5

Treatment of the results, according to Lineweaver & Burk (1934), does not give as clear a result as in the case of the decarboxylation of glutamate. Whilst the plot of $1/S$ against $1/v$ is a straight line for extract with cetavlon, that for the extract alone gives an S shaped curve. If the results for extract alone are plotted in the form $1/v$ against $1/S^2$ the line is straight. With a single enzyme this result suggests that either a ternary collision was involved or that an inhibitor was present.

The effect of cetavlon on other enzyme systems. No acceleration of any other enzyme system has been found. The systems tested and the methods of testing are summarized in Table 6.

DISCUSSION

The acceleration by cetavlon of the decarboxylation of glutamate and glutamine, which had previously been reported with intact cells (Krebs, 1948), has now been shown to take place with extracts of *Cl. welchii*, *Proteus morganii* and *Esch. coli*. The effects are generally of the same order as with intact cells, thus the main action cannot be due to an effect on permeability of the cell walls. At low substrate concentrations the degree of acceleration with intact cells was slightly higher than with extracts, and this may be due under these conditions to an additional effect of cetavlon on cell permeability.

Examination of the cetavlon effect at varying substrate concentrations showed that the detergent increased the apparent affinity of the decarboxylase for glutamate. Changes in the affinity of an enzyme for its substrate can be brought about by the

Table 6 *The effect of cetavlon on the reaction rates of enzymes other than glutamic decarboxylase and glutaminase of Clostridium welchii*

Name of enzyme	Source of enzyme	Method of preparation and experimental conditions	pH at which the system was tested	Effect of cetavlon (0.0025 M and 0.01 M final concentration)
Ornithine decarboxylase	Washed intact cells of <i>Cl. septicum</i>	Gale (1947)	5.5, 5.0, 4.0	None
Tyrosine decarboxylase	Washed intact cells of <i>Strep. faecalis</i>	Gale (1947)	5.5, 5.0, 4.0	None
Tyrosine decarboxylase	Extract of acetone dried cells of <i>Strep. faecalis</i>	Epps (1944)	5.5, 5.0, 4.0	None
Arginine decarboxylase	Washed intact cells of <i>Esch. coli</i>	Gale (1947)	5.0, 4.0	None
Arginine decarboxylase	Extract of <i>Esch. coli</i> disintegrated by grinding glass	Tested as with intact cells	5.0, 4.0	None
Histidine decarboxylase	Washed intact cells of <i>Cl. welchii</i> strain (6785)	Gale (1947)	5.0, 4.0, 3.0	None
Lysine decarboxylase	Washed intact cells of <i>Bact. cadaveris</i>	Gale (1947)	5.0, 4.0	None
Glutamic decarboxylase	Extracts of carrots and squash	Schales, Mims & Schales (1946)	5.0, 4.0, 3.0	None
Urease	Extract of Jack bean meal		5.0, 4.0	None

following mechanisms (a) increased concentration of substrate in the vicinity of the enzyme (Van Slyke, 1942), (b) an alteration in the properties of the enzyme by reaction with an added substance, (c) removal of an inhibitor

It seems unlikely that mechanism (a) is involved, as transport of material by surface active agents has been demonstrated only in the case of lipophilic substances (Dean & Vinograd, 1942), and neither glutamate nor glutamine is lipophilic

The reaction of surface active agents with enzymes usually leads to inactivation of the enzyme (Putman, 1948, Valko, 1946). On the other hand, Bodin & Hill (1945) found that the addition of anionic detergents to prototyrosinase increases the activity, they suggested that the increase was due to formation of enzyme material from a precursor, but did not investigate the effect of detergents on the affinity of the enzyme

The data on the effect of cetavlon at varying substrate concentrations strongly suggest that a competitive inhibitor of the glutamic decarboxylase is removed by cetavlon, either by solubilization of the inhibitor on micelles (for this effect see Alexander & Trim, 1946), or by combination with the inhibitor to form a complex

The experiments on the effect of cetavlon on the apparent affinity of the glutaminase do not give such a clear picture as those on the glutamic decarboxylase. However, the results suggest that cetavlon accelerates the glutaminase by the removal of an inhibitor

SUMMARY

1 Cell free extracts containing glutamic decarboxylase and glutaminase have been prepared from *Clostridium welchii*, *Proteus morganii* and *Escherichia coli* by grinding the cells with powdered glass, adding buffers to the ground mass and centrifuging

2 Of the original decarboxylase of the intact cells, about 60% was recovered in extracts from *Cl. welchii*, 30–40% from *Proteus morganii* and 25–40% from *Esch. coli*. The glutaminase activity of extracts was between 50 and 100% of that of the intact cells

3 The activity both of the decarboxylase and of the glutaminase in the extracts was increased by the addition of cetavlon

4 The acceleration of the decarboxylase by cetavlon in intact cells and extracts of *Cl. welchii* became smaller as the substrate concentration was raised, i.e. the apparent affinity of the decarboxylase for glutamate seems to be increased by the addition of cetavlon

5 Bacterial decarboxylases acting on ornithine, tyrosine, arginine, lysine and histidine are not accelerated by the addition of cetavlon, nor is the glutamic decarboxylase of *Daucus carota* (carrot) or *Cucurbita pepo* (squash)

The author wishes to express his thanks to Prof H. A. Krebs, F.R.S., for his help and criticism, and to Miss E. Ellis for technical assistance

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Factors Influencing the Polysaccharide Content of *Escherichia coli*

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(Received 28 February 1949)

It is known that bacteria build up reserves of polysaccharides within the cell, but little work has been done on the factors which influence their accumulation. Our object was to investigate factors influencing polysaccharide storage and the role which storage plays in the metabolism of the bacterial cell. Dawson & Hapgood (1943) reported that D phenylalanine caused increased storage of polysaccharide when *Escherichia coli* was grown on a glucose, but not on a mannose, medium. Gale (1947) has stated that polysaccharide formation occurs within the cell when *Esch. coli* is allowed to metabolize glucose in excess, but this material is itself metabolized as soon as the external glucose is exhausted. To investigate this, storage was examined where onset of the stationary phase was determined by both glucose and nitrogen exhaustion in the medium.

Additional interest in variations of polysaccharide content was occasioned by frequent observations that a fall in bacterial mass, as indicated by turbidity measurements, occurred in the early stationary phase. Monod (1942) also observed decrease in turbidity, attributed to a decrease in cell size, in this phase, and it was of interest to see whether this was related to polysaccharide content.

EXPERIMENTAL

The organism used throughout this work, unless otherwise stated, was no. 5928 of the National Collection of Type Cultures.

Growth cycles were followed turbidimetrically, using a Spekker photoelectric absorptiometer with neutral filters, H 508, and microcells. Since we have shown that values for the polysaccharide content are dependent on the stage reached in the stationary phase we have been careful to ensure that samples were taken at the same phase of growth. In every experiment the inoculum has been taken from a medium containing the same constituents as the medium to be used in the experiment, and from the early stationary phase, under which conditions minimum lag is obtained (Hinschelwood & Lodge, 1943).

Flasks were incubated in a thermostat at 38°. Determinations of pH were made with a Marconi type TF 717A pH meter. Dry weight determinations were made on 1 ml bacterial suspension placed on a watch glass and heated overnight in an electric oven (80–90°) to constant weight.

For polysaccharide determinations portions of at least 250 ml culture were centrifuged and the supernatant reserved for pH and sugar determinations. The cells were twice suspended in distilled water and again centrifuged, taken up in 10 ml distilled water, filtered through glass wool and used for polysaccharide and dry weight determinations. No sugar was detected in the supernatant after final centrifugation.

Media

Medium A, a simple salt medium containing 5.4 g KH_2PO_4 , 1.2 g $(\text{NH}_4)_2\text{SO}_4$, 6.6 g glucose and 0.66 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH adjusted to 7.12 with NaOH.

Medium B, a complex amino acid medium containing 0.1 g L-cystine, 0.5 g glycine, 1 g DL-valine, 5 g sodium glutamate, 15 g Nalactate, 5 g NaCl, 2.5 g $\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$, 0.35 g KH_2PO_4 , 0.30 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, pH adjusted to 7.5 with NaOH.

Addition of sugar was made separately. The medium and sugar were sterilized separately by boiling on successive days.

Determination of bacterial polysaccharide

The polysaccharide content of *Esch. coli* has been determined by Dawson & Haggold (1943), using the Sahyun (1931) method for the determination of tissue glycogen. Since this involves treatment with 10 N KOH in a boiling water bath the method appeared open to objection on the grounds that this would result in loss of such constituents as amino sugars. Furthermore, adsorption on charcoal is also employed and this may result in low recoveries. The determination of reducing sugar by the Hagedorn & Jensen (1923) technique, which is not specific for reducing sugar, is also open to objection. These possibilities were, therefore, investigated.

To determine reducing sugar the method of Somogyi (1937) was adopted. This reagent was found to keep well, and periodic restandardization revealed no significant change in reduction equivalent. The hydrolysis of bacterial polysaccharide was effected with H_2SO_4 and optimum conditions for this, and the effect of alkali treatment, were investigated.

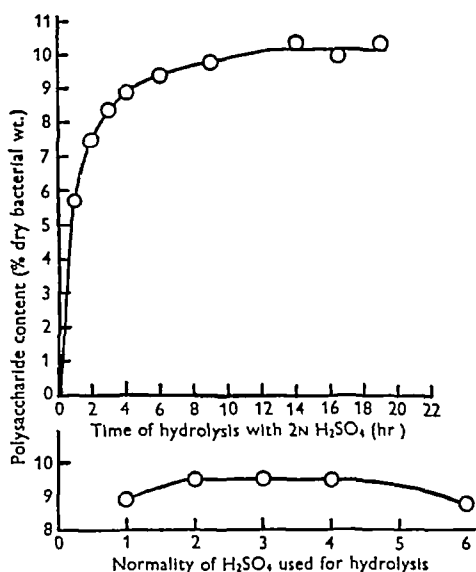


Fig 1 Polysaccharide content of *Esch. coli* determined by the Somogyi (1937) method, after varying periods of hydrolysis of washed cell suspensions with 2N H_2SO_4 , and after 16 hr hydrolysis of washed cell suspensions with varying concentrations of H_2SO_4 .

Effect of length of hydrolysis on polysaccharide determination

Portions (5 ml) of a bacterial suspension were hydrolysed in sealed ampoules with 2N H_2SO_4 for varying periods in a boiling water bath, the contents transferred quantitatively, neutralized and made to 50 ml. Portions (5 ml) were used for reducing sugar determinations (triplicate). The polysaccharide content of the organism is expressed in terms of glucose equivalents as a percentage of the dry bacterial weight. Fig 1 shows that hydrolysis is complete at approximately 10 hr. Accordingly, for convenience, all hydrolyses were carried out overnight.

Effect of acid strength on polysaccharide determination

Fig 1 shows the variation in polysaccharide content obtained when hydrolysis of the bacterial suspension was carried out with various acid strengths. It will be seen that there is little difference over the range 1–6N, maximum effect being obtained with 2–4N acid. Accordingly, we decided upon 2N acid for hydrolysis in all subsequent work.

Effect of pre treatment with alkali. The effect of treatment with 2N NaOH prior to acid hydrolysis was investigated. Portions (5 ml) of bacterial suspensions were made 2N with respect to NaOH, and the unsealed ampoules covered with glass bulbs and placed in the boiling water bath for given periods. They were then removed, made 2N with respect to H_2SO_4 , sealed and returned to the water bath. Treatment with 2N NaOH for 2 hr prior to 10 hr hydrolysis with 2N H_2SO_4 gave a polysaccharide content of 7.1% dry bacterial weight as compared with 15.5% with the control. Pre treatment with NaOH therefore results in a much lower figure for polysaccharide content.

Comparison of polysaccharide determinations by the Somogyi and Hagedorn & Jensen methods. For convenience, hydrolysis in this experiment was conducted for 2 hr only, and then the reducing sugar liberated determined by both the Somogyi (1937) and Hagedorn & Jensen (1923) methods. In three different experiments the latter method gave much higher figures, in some cases double the Somogyi value viz Somogyi 3.5, 2.7, 3.7 and Hagedorn & Jensen 7.3, 5.3 and 5.6% dry bacterial weight respectively. Clearly, the Hagedorn & Jensen method is determining reducing substances other than sugars. The findings that alkali causes destruction of approximately 50% bacterial polysaccharide, and that the Hagedorn & Jensen method of reducing sugar determination gives very high results with bacterial hydrolysates, was fully borne out when a direct comparison of Dawson & Haggold's (1943) method was made with our own (see p 333).

Comparison of polysaccharide determinations by the Dawson & Haggold technique and by acid hydrolysis. Seeking to explain the difference in observations recorded by Dawson & Haggold (1943) using the Sahyun (1931) method, and ourselves, employing acid hydrolysis, on the effect of D phenyl alanine, we undertook a direct comparison of both methods and also carried out the reducing sugar determinations by the Hagedorn & Jensen and the Somogyi techniques. Thus a comparison was afforded of both important stages in the procedure: first hydrolysis and then reducing sugar determination.

Two flasks were set up each containing 1 l of medium B to which 1% (w/v) of glucose and DL-tryptophan was added. D Phenylalanine (0.13 g) was added to one of these flasks and both inoculated with two loops of bacterial suspension. Polysaccharide determinations were carried out, when the stationary phase had been reached, by both methods. The results (Table 1) confirm our previous findings, namely, that destruction of 40–50% of bacterial polysaccharide is brought about by the KOH treatment in the Sahyun method, and also that the Hagedorn & Jensen method of sugar determination gives results which are about 50% too high, presumably due to the presence of reducing substances other than sugars. Quite clearly the Sahyun method for glycogen is not suitable for total bacterial polysaccharide determinations, and neither is the Hagedorn & Jensen method suitable for the determination of reducing sugar liberated from such material. All our work therefore has, with justification, been based on acid hydrolysis of the cells followed by determination of reducing sugar by the Somogyi method.

It will be noted that, using the Sahyun and Hagedorn & Jensen methods, the polysaccharide content is slightly higher in the presence of D phenylalanine. However, using the method of Dawson & Happold, the polysaccharide content obtained is very much greater than that recorded by them (about 5%) and this is apparently due to a strain difference. To investigate this possibility the polysaccharide content of various strains has been examined (see p. 335).

Table 1 Comparison of polysaccharide determinations by the Sahyun and acid hydrolysis methods with reducing sugar determinations by the Hagedorn & Jensen and Somogyi techniques

Method of hydrolysis	Polysaccharide content (% dry bacterial wt)			
	Sahyun		2 N H ₂ SO ₄	
	Hagedorn & Jensen	Somogyi	Hagedorn & Jensen	Somogyi
Control	15.5	10.8	28.2	19.5
0.013% (w/v) D phenylalanine	17.5	12.8	26.8	18.7

Accuracy of the method. Two sets of duplicate determinations were carried out, with different dry weights, hydrolyses and reducing sugar determinations being all carried out independently, all possible experimental variables were introduced. For two different samples of *Esch. coli* grown under different conditions the polysaccharide contents (% dry bacterial weight) were sample 1—13.5, 13.3, sample 2—17.5, 17.4.

Final method for bacterial polysaccharide determinations. Portions (5 ml.) of bacterial suspension (washed free from sugar present in medium as previously described) were placed in ampoules, 0.3 ml. conc. H₂SO₄ added to each to make 2 N with respect to acid, and the ampoules sealed and placed in a boiling water bath overnight. They were then opened and the contents transferred quantitatively, with filtering to remove debris, to 50 ml. graduated flasks, neutralized and made to the mark with water. Portions (5 ml.) were used in triplicate for the determinations with 5 ml. Somogyi reagent. Liberated I₂ was titrated with 0.005 N Na₂S₂O₃, which was made from 0.1 N stock solution immediately before use.

RESULTS

The variation of polysaccharide content of Escherichia coli at various phases of the growth cycle

Variations in polysaccharide content with time. Growth was followed by turbidity measurements in a medium in which glucose was known to be in excess (simple medium A). The fluctuation of polysaccharide content with time is shown in Fig. 2. It is seen that between 20 and 38 hr. a fall in polysaccharide occurs. In another experiment the polysaccharide content during this phase of growth was followed more fully over a period of 46 hr. The polysaccharide content 1.75 hr. before onset of the stationary phase was 16.9%, 22 hr. after onset it

was 12.2% and 42 hr. after, 9.0%. A fall in polysaccharide content over long periods of the stationary phase has been confirmed in several subsequent experiments concerned with the influence of other factors.

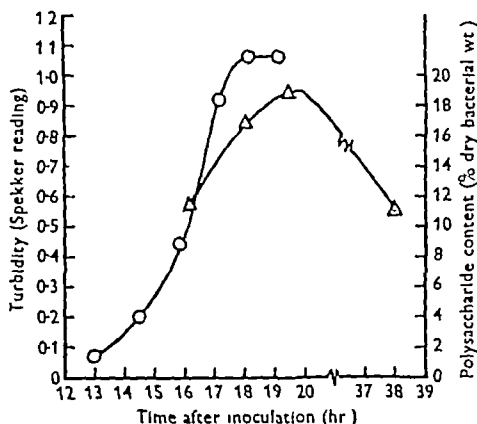


Fig. 2 Fluctuation of polysaccharide content of *Esch. coli* with time. Growth cycle followed turbidimetrically (O—O), and polysaccharide content (Δ—Δ) determined at various stages of growth and stationary phase.

Influence of factors determining onset of the stationary phase. Using the simple medium A, glucose concentration was maintained at 20 g/l and the concentration of (NH₄)₂SO₄ varied. A linear relationship was obtained between stationary population and (NH₄)₂SO₄ concentration up to 1.2 g/l, the highest concentration used, at which the stationary population was approximately 10⁹ bacteria/ml. Similarly, with excess (NH₄)₂SO₄ present at a concentration of 4 g/l, a linear relationship existed up to the highest concentration of glucose used (0.9 g/l) and the stationary population was approximately 3.7 × 10⁸ bacteria/ml. These findings are in accordance with those of Dagley & Hinshelwood (1938) and Hinshelwood & Lodge (1939) for *Aerobacter aerogenes*, and indicate that over these ranges of foodstuff concentration the onset of stationary phase is determined by exhaustion of foodstuff and not by adverse conditions or toxic products. This was confirmed by following the consumption of glucose in the case of a medium for which glucose exhaustion during growth was anticipated and one for which glucose was in excess of growth requirements (Fig. 3).

In Fig. 3 the fall in turbidity in the stationary phase is clearly shown. From this information it was possible to follow the storage of polysaccharide during the growth of *Esch. coli* when, on one hand, glucose exhaustion determined onset of the stationary phase and, on the other hand, when the glucose was in excess of requirements. Results of these experiments are given in Table 2. It is evident that

the fluctuations of polysaccharide content in the stationary phase are not determined by glucose exhaustion

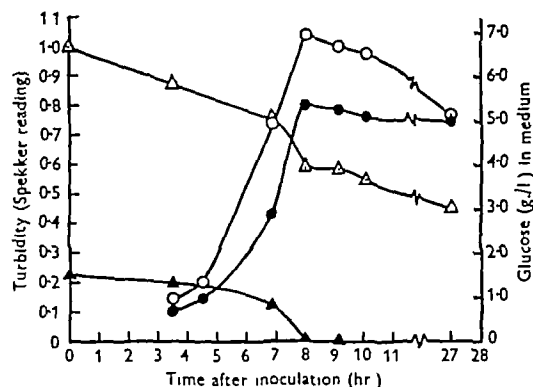


Fig 3 Relation between growth of *Esch. coli* and amount of glucose in the simple ammonium salt-glucose medium A (a) Excess glucose present growth (O—O) glucose content of medium during growth (Δ—Δ) (b) Limiting concentration of glucose present growth (●—●), glucose content of medium during growth (▲—▲)

Cessation of cell division and polysaccharide storage When the stationary phase is reached the total bacterial mass, as measured by turbidity, does not remain constant, but invariably shows a steady decline. We compared the fall in polysaccharide content during the stationary phase with this fall in bacterial mass. It is seen, however, from Table 2 that storage of polysaccharide has not in every case reached its maximum when division ceases, and from Fig. 2 it is also seen that the maximal storage does not exactly coincide with the beginning of the stationary phase. Thus decrease in bacterial mass cannot be correlated with fall in polysaccharide content.

The effect of D phenylalanine on polysaccharide content of *Escherichia coli*

Results using the method of acid hydrolysis Dawson & Hapgood (1943), using the Jebbs strain of *Esch. coli*, reported that 0.01% (w/v) DL-phenylalanine (but not the L-isomer) caused an increase in cell polysaccharide grown in the

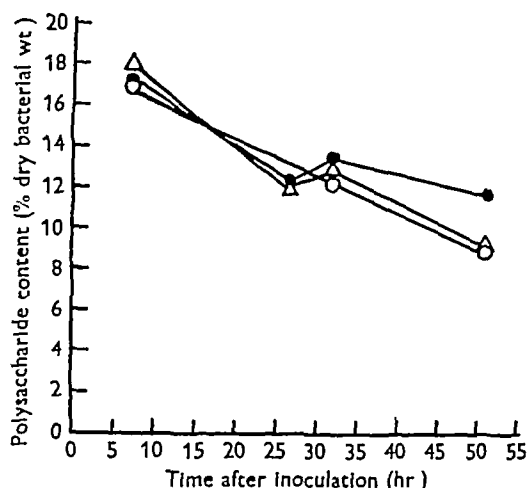


Fig 4 Polysaccharide content of *Esch. coli* grown in presence and absence of D phenylalanine on simple ammonium salt-glucose medium A, control, O—O, 0.013% (w/v) D phenylalanine added before inoculation, ●—●, and 0.013% (w/v) D phenylalanine added when stationary phase reached (7.25 hr), Δ—Δ

amino acid medium B. We sought to confirm this observation using both the simple and complex media and using the present method of polysaccharide determination. Of three flasks containing 1500 ml simple medium A, 0.013% (w/v) D phenylalanine was added to one, and growth curves were determined. To another, 0.013% (w/v) D phenylalanine was added when the stationary phase was reached. Samples

Table 2 Effect of limiting concentration of glucose and ammonium sulphate on stationary population and polysaccharide content of *Escherichia coli*

Glucose (g /l.)	(NH ₄) ₂ SO ₄ (g /l)	Stationary population (10 ⁸ /ml)	Time after reaching stationary phase (hr)	Polysaccharide content (% dry bacterial wt)
(a) Onset of stationary phase determined by glucose exhaustion				
1.50	5.0	725	0	9.7
			19	13.4
			24	8.8
2.25	5.0	925	0	12.3
			19	12.2
			24	10.2
(b) Onset of stationary phase determined by (NH ₄) ₂ SO ₄ exhaustion				
5.0	1.2	737	0	14.0
			19	14.7
			24	11.1
5.0	1.8	950	0	11.6
			19	16.5
			24	11.5

(350 ml) of cultures were withdrawn at intervals, the polysaccharide content of the cells determined, and these are shown in Fig 4. D-Phenylalanine has no effect on growth rate or polysaccharide storage in this medium. The decline in polysaccharide content previously noted is shown in each case. The experiment was repeated using an inoculum of which about twenty five generations of the cells had been grown in the medium containing 0.013% (w/v) D-phenylalanine. Here again there was no significant difference between cultures containing D-phenylalanine and those without.

Using the amino acid medium B, to which glucose was added to a concentration of 1% (w/v), polysaccharide contents were estimated when turbidities showed in each case that the culture had just attained its stationary phase. Besides adding D-phenylalanine to two of the four flasks, 1% (w/v) tryptophan was also added to two of the flasks since this was present in the medium used by Dawson & Hapgood (1943). The results obtained again show no increase in polysaccharide content due to the presence of phenylalanine, e.g. control, 22.2%, 1% (w/v) DL-tryptophan added, 21.5%, 1% (w/v) DL-tryptophan + 0.02% (w/v) D-phenylalanine, 20.3% and 0.02% D-phenylalanine, 19.5% polysaccharide dry bacterial weight.

Polysaccharide content of various strains of *Escherichia coli*. Five different strains of *Esch. coli* were obtained through the courtesy of Dr D. E. Dolby of the Bacteriology Department of this University. They were grown in medium B containing 1% (w/v) glucose, and their polysaccharide contents determined in the usual way. It was found that there is a quite wide variation with strain, but none is as high as the strain used in this work. The polysaccharide contents were as follows: NCTC no 5928, 21.2%; coli I, 10%; coli B, 6.9%; coli D, 9.5%; coli 4, 13.9% and coli 7148, 12.7%. The lowest storer was the 'B' strain which was a non-motile organism. It was decided, therefore, to see whether this organism (with a polysaccharide content similar to that of the Jebbs strain used by Dawson & Hapgood) showed an increased storage in the presence of D-phenylalanine, both in the presence and absence of glucose.

To four flasks containing 250 ml medium B additions were made as follows: (1) 0.33% (w/v) glucose, (2) 0.33% (w/v) glucose + 0.013% (w/v) D-phenylalanine, (3) none, (4) 0.01% (w/v) D-phenylalanine. The polysaccharide content (% dry bacterial weight) of the 'B' strain grown on these four media was found to be (1) 8.2, (2) 8.1, (3) 3.3, (4) 3.3. Hence it was concluded that D-phenylalanine has no effect on the polysaccharide storage of *Esch. coli*. It is of interest to note that with this low storing strain, as with the highest one, the addition of glucose to the medium causes a relatively large increase in stored polysaccharide. That low storage was not due to non-utilization of glucose was demonstrated by turbidity determinations during growth. The 'B' strain grew to approximately the same turbidity as the high storing NCTC strain no 5928.

Effect of various sugars on polysaccharide storage in the amino acid medium

Effect of sugar concentration. Dawson & Hapgood (1943) reported that mannose had little effect on the polysaccharide storage of *Esch. coli* on the amino acid medium B using the Jebbs strain of organism.

We have repeated their experiments and, in addition, investigated the effect of the sugar concentration with both mannose and several other sugars.

To flasks containing 250 ml amino acid medium B (pH 7.5) increasing amounts of a sterile sugar solution were added. Each flask was inoculated with two loops of a culture, which had grown on the amino acid medium containing the sugar in question, at a time when it had just attained its stationary phase. Growth was followed turbidimetrically and the cultures were centrifuged and washed when the stationary phase was reached. The effect of sugar concentration is given in Fig 5. It will be seen that

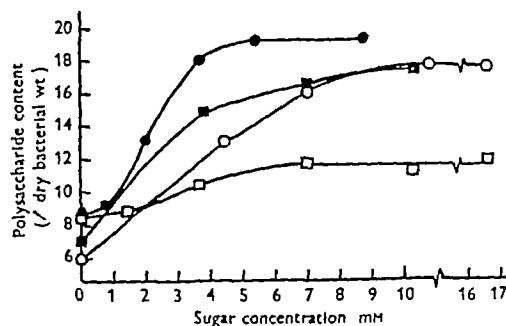


Fig 5. Relation between polysaccharide content of *Esch. coli* and concentration of sugar present in amino acid medium B, glucose, ○—○, lactose, ●—●, mannose, □—□, and galactose, ■—■.

a certain minimum storage of polysaccharide is obtained in the absence of sugar, but on the addition of it the stored polysaccharide increases to a maximum value, above which further additions of sugar produce no increase. It is also seen that, although some increase in storage was obtained when mannose and fructose (see Fig 6) were present, the increase was markedly less than in the case of glucose, galactose and lactose.

Effect of pH. Hinshelwood & Lodge (1939), working with *Bact. lactis aerogenes*, have shown that stationary population is determined by the pH of the medium as well as by foodstuff concentration. At adverse pH values on the acid and alkaline sides of neutrality the total growth, and hence the foodstuff consumption, is greatly reduced. Accordingly, we have investigated the effect of pH on polysaccharide storage since this appears to depend on the amount of sugar metabolized. For each sugar at a concentration of 3.3 g/l a range of media of pH between 6 and 8.5 was inoculated as before, the cells centrifuged when the stationary phase had just been reached and their polysaccharide content determined.

The dependence of polysaccharide content on pH for various sugars is shown in Fig 6, where the

dependence of storage on pH is seen to be greatest in the case of the sugars for which the storage is highest

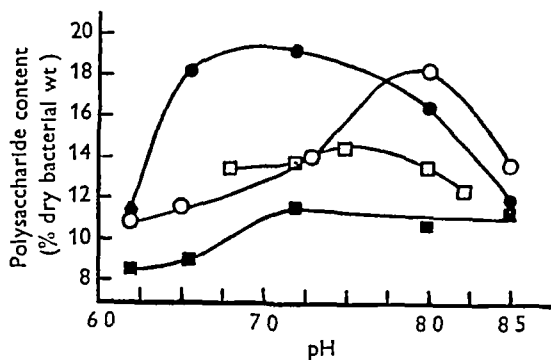


Fig 6 Effect of pH on polysaccharide content of *Esch coli* with various sugars at a concentration of 3 g/l in amino acid medium B, glucose, ○—○, galactose, ●—●, mannose, □—□, and fructose, ■—■

DISCUSSION

It is sometimes assumed that the function of storage of polysaccharide by the cell is to serve as a store of carbohydrate for use when the other sugars in the medium are exhausted. Although we have found that there is a decline in storage during the stationary phase this is not directly related to the exhaustion of the normal supply of carbohydrate in the medium. Whilst it is possible that the polysaccharide may serve as a reserve source of energy, an alternative explanation for the fall in polysaccharide content may be found in the fact that increasing numbers of cells are dying in the stationary phase, and the decrease is reflected in autolytic activity. We cannot conclude from our results that the process of storage ceases exactly at the same time as cell division, whether the latter is due to exhaustion of glucose in the medium or not. On these grounds it is difficult to ascribe the fall in bacterial mass as reflected in the turbidity of the culture, which Monod (1942) first reported, and which we have repeatedly confirmed, entirely to dissimilation of stored carbohydrate.

With the present strain of *Esch coli*, and using the method of estimation which we have developed, we have not observed any effect of small quantities of D-phenylalanine in increasing polysaccharide storage as reported by Dawson & Happold (1943). It would appear that the effect ascribed by Dawson & Happold to D-phenylalanine was, in fact, due to the method of estimation used. We conclude that the Sahyun (1931) method for glycogen determinations, coupled with reducing sugar determinations by the Hagedorn & Jensen (1923) technique, is unsuitable for bacterial polysaccharide determinations.

It is seen that although a low level of storage is obtained when the cells are provided with lactic

acid and various amino acids a greatly increased level is obtained when sugars are provided, particularly when pH is favourable to growth and storage. On the other hand, all sugars are not as effective as glucose or galactose, lower storage is obtained with mannose (which Dawson & Happold have previously commented upon) and fructose.

SUMMARY

1 The polysaccharide content of *Escherichia coli*, grown under various conditions, has been determined by acid hydrolysis of washed cell suspensions, followed by determination of reducing sugars by the method of Somogyi (1937).

2 The polysaccharide content falls during the stationary phase, but the onset of this fall does not exactly coincide with the cessation of cell division.

3 In a glucose ammonium salt medium this trend is obtained whether exhaustion of nitrogen or exhaustion of glucose determines the onset of the stationary phase.

4 For these reasons the fall is not attributed to the utilization of stored polysaccharide by the cells when the sugar in the medium is used up.

5 For lactose, galactose and glucose, grown at pH 7.5 in an amino acid medium which will support some growth in the absence of a sugar, the stored polysaccharide increases markedly with sugar concentration to a maximum, beyond which further additions cause no increase. With mannose and fructose little increase is produced.

6 At a fixed sugar concentration, variation of pH affected the storage of cells grown in glucose and galactose media. In these cases there are pH optima, which are not shown with the low storing sugars, mannose and fructose.

7 There is a wide variation of stored polysaccharide content for different strains of *Esch coli* grown under identical conditions.

8 The Sahyun (1931) method of glycogen determination, coupled with the Hagedorn & Jensen (1923) method for reducing sugars, is not satisfactory for the estimation of total bacterial polysaccharide.

9 The increased storage of polysaccharide, reported when this method of estimation was used and the cells were grown in the presence of D-phenylalanine, is not confirmed when the method described in paragraph 1 is employed.

We wish to thank Prof F C Happold, at whose suggestion the research was undertaken and from whose advice we have benefited at various stages in its progress. We are indebted to Dr H J Rogers who drew our attention to the advantages of the method of estimation for bacterial polysaccharide which we adopted, and indicated possible sources of error in the Sahyun (1931) method. We have also benefited from discussions with Mr E S Holdsworth, who is undertaking similar investigations with *Corynebacterium diphtheriae*.

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Breakdown of the Oxidized Forms of Coenzymes I and II by an Enzyme from the Central Nervous System

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(Received 31 March 1949)

The enzymic breakdown of cozymase in various cells, and especially in tissues from the central nervous system, attracted our attention as being an unexpectedly rapid reaction (McIlwain & Hughes, 1948, McIlwain & Rodnight, 1949). It appeared that the breakdown, if unimpeded, would rapidly deplete tissues of this very important metabolite. Certain factors which would partly restrain the degradation under physiological conditions were observed, but the problem of the normal function of such a potent system remained unanswered. In general terms this appeared likely to be one of participation in, or control of, reactions in which cozymase functioned as coenzyme, and because of the importance of these the degrading enzyme has been studied further.

organisms takes place at the pyridinium N (Fig 1, Handler & Klein, 1942, McIlwain & Hughes, 1948). In some other tissues different processes preponderate. A link between nicotinamide and a substituted ribose is at present known to exist naturally only in cozymase (Co I), coenzyme II (Co II) and nicotinamide mononucleotide, although other natural and synthetic products approach these structures in various ways. The possible action of the enzyme on a number of such substances has now been examined.

EXPERIMENTAL

Materials

Cozymase. The specimens described previously (McIlwain & Rodnight, 1949) were used.

Dihydrocozymase. The preparation followed Schlenk (1941). Reaction with a specimen of Co I (120 mg, containing 68 mg Co I) was complete in 30 min and differential ethanol precipitation yielded a first fraction of 44 mg dihydrocozymase. Titration with $K_3Fe(CN)_6$ 1.2 mg required 0.45 ml of 0.005N solution, content, 0.94 $\mu\text{mol/mg}$. Manometric reaction with $K_3Fe(CN)_6$ 4.3 mg gave 4.65 $\mu\text{mol CO}_2$, yield, 1.08 $\mu\text{mol/mg}$. Optical density at 340 m μ ($c=0.094$ mg/ml), 0.501, content, 0.86 $\mu\text{mol/mg}$. The pure material as disodium salt contains 1.41 $\mu\text{mol/mg}$.

Two other specimens, of 45 and 55% purity, were also examined.

Coenzyme II was prepared from liver (in different batches, sheep and horse livers were used) following in part Altman's (1948) adaptation of the procedure of Warburg, Christian & Griese (1935). After precipitation as Hg and Ba salts, materials up to 10% pure were obtained. We are indebted to Dr B C J G Knight (Wellcome Physiological Research Laboratories) for a supply of horse liver immediately after slaughter.

Dihydrocoenzyme II. Attempted preparation from a coenzyme specimen of 10% purity, by the method used for

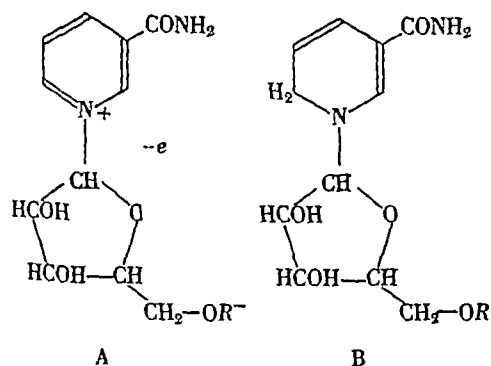


Fig 1 A, coenzymes I or II, e indicates point of fission by brain preparations, B, or an isomeride with differently placed double bonds, dihydrocoenzymes I or II.

It is fairly well established that the breakdown of cozymase in nervous tissues and in certain micro-

dihydrocozymase, was unsuccessful. Solutions of the substance were prepared enzymically, as described in the text below.

Nicotinamide glycosides We are greatly indebted to Prof A R Todd and Dr L J Haynes (University Chemical Laboratory, Cambridge) for specimens of these compounds in their dihydro forms. They were synthetic materials of which full details will be published elsewhere. Their oxidized forms were prepared by reaction with $K_3Fe(CN)_6$ in a manometric apparatus (see below).

Analytical methods

Ferricyanide and iodine titrations of dihydronicotinamide derivatives With I_2 , a 0.005 M solution in KI was used and titrations performed in dilute acetic acid containing starch. With $K_3Fe(CN)_6$, a 0.005 M solution was used, and the dihydro derivative was titrated in dilute acetic acid with KI and starch as external indicator.

Manometric reaction with ferricyanide (of Haas, 1937) The dihydronicotinamide derivatives were dissolved in 1 ml of 0.02 M $NaHCO_3$ saturated with 5% (v/v) CO_2 in N_2 in the main compartment of conical Warburg vessels of approx 15 ml. The side arms of experimental and control vessels contained 0.3 ml of 0.05 M $K_3Fe(CN)_6$ which was tipped into the main compartment after equilibration with the above gas mixture, reaction was usually complete in 1 hr. This method was used also for reoxidizing dihydrocozymase to cozymase for use as substrate, the resulting solution, containing also the ferri and ferro cyanides, was employed as it had been established that such concentrations of the reagents had no effect on cozymase breakdown.

Nicotinamide and acid formation during reactions with the coenzymes were determined as described previously (McIlwain & Rodnight, 1949).

Spectrophotometric methods The Beckman DU quartz spectrophotometer was used for determining dihydronicotinamide derivatives at concentrations of about 10^{-4} M and in 3 ml of aqueous solution. Horecker & Kornberg's (1948) value of 6.22×10^4 sq cm/mol was used for calculating concentrations of coenzymes I and II from the observed optical densities at 340 m μ . In demonstrating the stability of dihydrocozymase to the enzyme, reaction mixtures were examined between 320 and 400 m μ before and after an incubation which inactivated 1.5 μ mol cozymase in a control experiment. No change in the absorption of 1.6 μ mol dihydrocozymase was found.

Determination of coenzyme II This followed Adler, Euler, Günther & Plass (1939) and Ochoa & Weisz Tabori's (1948) use of the isocitric dehydrogenase of mammalian heart. Sheep heart was treated according to Straub's (1942) procedure, and the acetone powder kept dry in a refrigerator. Portions of 0.5 g of this were extracted weekly with 10 ml of phosphate buffer (pH 7.3), the extract dialysed, kept cold, and used as isocitric dehydrogenase. DL isocitric acid was prepared according to Fittig & Miller (1898) and Nelson (1930).

In the determination, the following solutions were made to 2.95 ml in spectrophotometer cells: 0.1 M veronal buffer (pH 7.2), 1.0 ml, 0.01 M $MnCl_2$, 0.2 ml, the Co II containing solution and the dehydrogenase (0.05 ml). The optical density at 340 m μ was measured at room temperature (approx 20°), readings being taken until values were steady, and 0.05 ml M isocitrate at pH 7.2 was then added. Readings were again taken, and from the change the volumes and the

known extinction coefficient of dihydrocoenzyme II, its concentration was calculated. The course of an experiment of this type is illustrated in Fig 2.

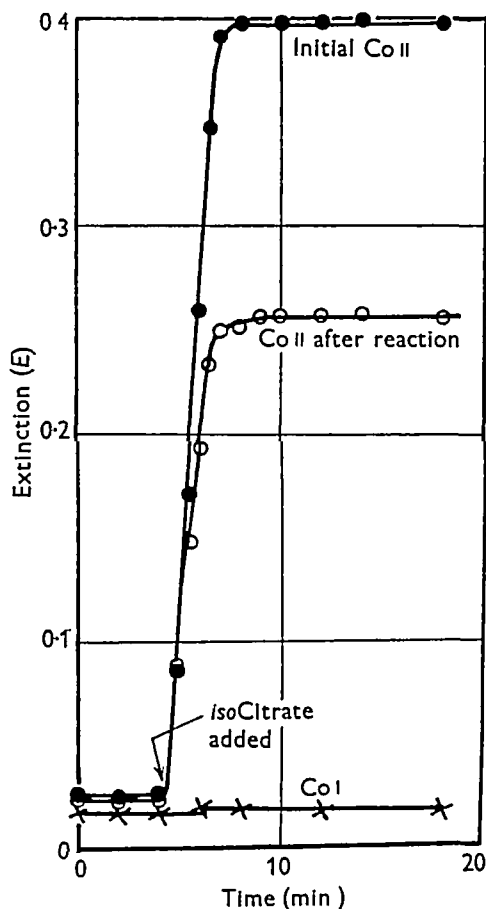


Fig 2 Use of isocitrate system to show degradation of coenzyme II by a brain preparation. The initial and final Co II solutions (one sixth of reaction mixture) are shown by the graph to contain respectively 0.86 and 0.56 μ mol of the coenzyme. The result with cozymase was from a vessel containing 1 μ mol cozymase, similarly diluted, and illustrates the specificity of the isocitric system and the purity of the cozymase.

Preparations of the cozymase degrading enzyme

Tissue suspensions were made by grinding with sand, or by homogenizing, and were washed with saline. Aqueous extracts of washed tissue were made by repeated extraction of such suspensions with water. These have been described by McIlwain & Rodnight (1949). Preparations *SsDC* were made by extracting suspensions with strong salt solutions, dialysing and centrifuging. Data concerning them are given in the descriptions of the experiments in which they were used.

RESULTS

Dihydrocozymase

No change has been observed to be caused in dihydrocozymase by various brain preparations, and its much greater stability than cozymase in

nervous tissue is therefore noteworthy. The analytical methods used to detect possible change would in several cases have detected a reaction with a velocity less than 1% of that observed with cozymase. Table 1 shows that no reaction leading to liberation of nicotinamide was detectable, nor one leading to the formation (as with cozymase) or absorption of acid. Moreover, dihydrocozymase itself was determined in certain reaction mixtures, before and after incubation, by its absorption band at $340\text{ m}\mu$, this showed less than 3% change. The dihydrocozymase was not pure, but three independently prepared specimens from three specimens of cozymase were examined. The initial cozymase specimens were degraded by brain preparations. Also, in one case the dihydrocozymase which had failed to react was reoxidized by ferricyanide and the resulting cozymase found to act as substrate. The dihydrocozymase specimen which failed to react did not inhibit the reaction with cozymase. Lack of reaction with dihydrocozymase thus cannot be attributed to effects of associated materials.

of whole brain tissue which had been ground with sand and thoroughly washed with 0.9% sodium chloride. It was, however, unstable in the presence of homogenized whole brain or blended heart muscle, aerobically, when oxidation to cozymase itself could be expected.

Nicotinamide glycosides

The most interesting of these compounds was the naturally occurring nicotinamide ribofuranoside. This contains the nicotinamide ribose link which in cozymase is split during the present reaction. Nicotinamide riboside itself was, however, stable to the brain preparations used. Any reaction with three different enzyme preparations was of less than 1% of the speed with cozymase (Table 1). As criteria of reaction, appearance of nicotinamide (Koenig reaction) and acid formation were used.

In view of the stability of the riboside, it was not surprising that compounds less closely related to cozymase did not act as substrates. The mannoside, galactoside and glucoside were examined by the

Table 1 *Possible liberation of nicotinamide from its derivatives by brain preparations*

(All enzyme preparations were of ground tissues washed with and diluted in 0.9% NaCl. Rates were determined from manometric observation of the course of evolution of CO_2 from bicarbonate containing solutions, and checked by nicotinamide estimation at the end of the reactions. The possible reaction with dihydrocozymase was also examined, with similar results, with two other preparations of grey and white matter from sheep brain, one from guinea pig brain, and one from ox spinal cord. The riboside was examined also with preparations from white matter (sheep) and spinal cord (ox), and the mannoside, glucoside and galactoside with another preparation in addition to that quoted below, results were the same.)

Potential substrate ($\text{M} \times 10^{-4}$)	Source of enzyme preparation	Rate of reaction ($\mu\text{mol/mg/hr}$)
Cozymase (5)	Whole sheep brain	0.95
Dihydrocozymase (5)	Whole sheep brain	<0.03
Cozymase (5)	White matter from centrum ovale of sheep brain (preparation A)	0.92
Dihydrocozymase (5)	White matter from centrum ovale of sheep brain (preparation A)	<0.008
Dihydrocozymase (5) with ferricyanide (11)	White matter from centrum ovale of sheep brain (preparation A)	0.84
Cozymase (3.8)	White matter from centrum ovale of sheep brain (preparation B)	0.96
Dihydrocozymase (4)	White matter from centrum ovale of sheep brain (preparation B)	<0.007
Dihydrocozymase (4) with cozymase (3.8)	White matter from centrum ovale of sheep brain (preparation B)	0.94
Cozymase (5)	Whole sheep brain	0.88
Nicotinamide ribofuranoside (16)	Whole sheep brain	<0.007
Nicotinamide mannoside (10)	Whole sheep brain	<0.007
Cozymase (5)	Sheep brain, white matter	1.13
Nicotinamide glucoside (10)	Sheep brain, white matter	0.01
Nicotinamide galactoside (10)	Sheep brain, white matter	<0.01

Dihydrocozymase added to brain preparations was found to be stable not only anaerobically, under which conditions most of the present reactions were carried out, but also aerobically when in the presence

methods of Table 1, and any reaction found to be less than 1% of that with cozymase. Dihydroderivatives of all four compounds were also examined, with a similar result.

Coenzyme II

Thus was found to be acted on by the enzyme only slightly less rapidly than cozymase. The reaction was first followed in bicarbonate buffered mixtures at

The rate of reaction was also followed by determination of the coenzyme II at different times during the reaction. Fig. 2 shows the determination of coenzyme II in an experiment in which this was done. Warburg vessels contained in their main compart-

Table 2 Breakdown of coenzyme II by brain preparations

(Reaction mixtures contained the coenzymes (1 μmol), NaHCO_3 (22.5 μmol), NaCl (155 μmol) and brain preparations in 2.5 ml, with 5% (v/v) CO_2 in air at 37° . At intervals portions were removed, the reaction stopped with Zn salts, and nicotinamide determined as described by McIlwain & Rodnight (1949). The extract *SsDC* contained 1.2 mg protein/ml and 0.5 ml was used in each experiment.)

Co II content of preparation ($\mu\text{mol}/\text{mg}$)	Brain preparation	Rate of breakdown of coenzyme II	
		($\mu\text{mol}/\text{mg}/\text{hr}$)	(% of rate of cozymase in same experiment)
0.056	Guinea pig, mixed tissue ground and washed with NaCl	0.34	89
0.056	Ox spinal cord, as above	0.51	71
0.087	Sheep brain extract <i>SsDC</i>	1.40	77
0.087	Sheep brain extract <i>SsDC</i>	1.62	85

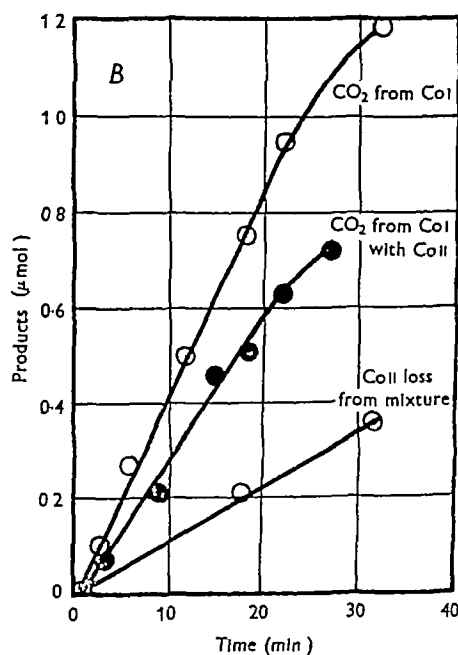
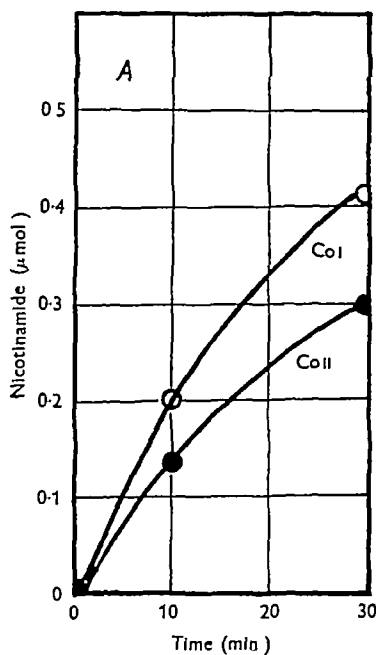


Fig. 3 Kinetics of coenzyme breakdown by brain preparations. A, nicotinamide liberated from coenzymes I and II, each 1 μmol , in 2.5 ml NaCl NaHCO_3 N_2/CO_2 (pH 7), by ground washed sheep spinal cord (equivalent to 8.3 mg dry wt) at 37° . B, CO_2 evolved during breakdown of cozymase (1.3 μmol) in 1.4 ml NaCl NaHCO_3 N_2/CO_2 (pH 7) and of a mixture of cozymase and coenzyme II (total 1.3 μmol , in ratio 3/4) by a similar preparation of the degrading enzyme from ox spinal cord. Loss of coenzyme II from the same mixture is also shown.

pH 7 by liberation of nicotinamide from a preparation containing about 4% of the coenzyme, and the rate of reaction found to be approx. 80% of that with cozymase. Purer coenzyme specimens, and various preparations of the enzyme, gave values between 70 and 85% of the rate with cozymase (Table 2, Fig. 3).

ments 0.9% NaCl and 0.01 M- NaHCO_3 (1 ml) with a brain preparation *WS* (a sodium sulphate precipitate of a water extract of sheep brain washed with 0.9% NaCl , with activity of 25 μmol cozymase degraded/hr/mg N). The coenzyme II (1 $\mu\text{mol}/0.2$ ml, as material of 9% purity) was

contained in a side arm, yellow phosphorus in a centre well, and 5% (v/v) CO_2 in N_2 in the gas space. Other vessels contained cozymase, and others no substrate. After equilibration at 37° , reaction was started by tipping. Carbon dioxide evolution was followed and vessels removed at different times and put in ice water. To the portions taken for coenzyme II determination, $\frac{1}{3}$ vol of 0.5 M nicotinamide was added and specimens taken for test with the isocitric system. From determinations which included that of Fig. 2, the rate of breakdown of coenzyme II was found to be 76% of that of cozymase.

The simultaneous degradation of the two coenzymes in a mixture containing them both in similar concentrations is shown in Fig. 3B. The results indicate that the two coenzymes compete for the degrading system.

Dihydrocoenzyme II

Demonstration of the stability of dihydrocoenzyme II in the presence of the enzyme was carried out as follows.

To 9.85 ml of solution containing $1.16 \mu\text{mol}$ Co II and the MnCl_2 and veronal buffer (0.1 M) of the isocitric system in a glass stoppered tube, was added 0.15 ml of the heart isocitric dehydrogenase. Sodium DL-isocitrate (0.1 M, 0.025 ml) was added, and the optical density of a portion of the solution followed in a closed cell at $340 \text{ m}\mu$. Another cell containing all reagents but no Co II was used as control. In 16 min at room temperature the optical density became almost steady at a value corresponding to $1.06 \mu\text{mol}$ of dihydrocompound, a further 0.005 ml of the isocitrate was added, and the reading rose to correspond to $1.11 \mu\text{mol}$. The whole solution was then heated quickly to 100° for 1 min, cooled in running water and centrifuged. The optical density of the solution compared with that of the control similarly treated fell by 10%, presumably due to the known instability of the dihydro compound. Samples of this solution were used as source of dihydrocoenzyme II in the following experiment.

Spectrophotometer cells were prepared of which three contained the foregoing reaction mixture (2 ml.). To one of these was added 1 ml. of water, to two others a preparation *SsDC* of the cozymase splitting enzyme from ox cord, as a clear solution. Another cell which served as control in the spectrophotometer contained the control solution of the previous paragraph (2 ml.) with the enzyme. Other cells contained the same control solution, enzyme and cozymase ($0.27 \mu\text{mol}$). Change in the latter mixtures was determined by estimating nicotinamide, change in the former by spectrophotometric estimations at $340 \text{ m}\mu$.

The results (Fig. 4) showed that the system rapidly inactivated cozymase (rate greater than $0.55 \mu\text{mol/hr}$), but caused very little change in dihydrocoenzyme II. The latter change in both control and experimental vessels was of about $0.02 \mu\text{mol/hr}$, any difference between these two due to an action of the enzyme itself on dihydrocoenzyme II was of less than $0.005 \mu\text{mol/hr}$, i.e. less than 1% of the rate of reaction with cozymase. The small change observed

in all vessels containing the dihydrocompound was presumably due to its instability in the reaction mixture in air at pH 7.2, no attempt had been made to remove dissolved oxygen initially present. In a vessel containing the enzyme in 3 ml with both dihydrocoenzyme II ($0.28 \mu\text{mol}$) and cozymase ($0.18 \mu\text{mol}$), the dihydrocompound underwent no change other than that of the control, while cozymase was decomposed rapidly (Fig. 4). The experiment

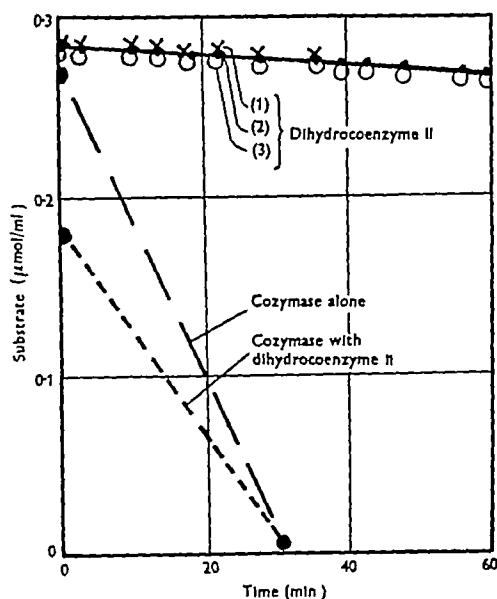


Fig. 4 Stability of dihydrocoenzyme II in the presence of the enzyme degrading cozymase. Above dihydrocoenzyme II determined spectrophotometrically in three solutions (indicated by different points) which contained (1) no further additions, (2) and (3) the ox spinal cord preparation *SsDC* (1.5 mg dry weight). Below change in cozymase with the same quantity of cord preparation, with and without dihydrocoenzyme II.

was not adequate to give comparison of the rates of reaction with cozymase in the presence and absence of dihydrocoenzyme II, but these could not have differed greatly. Therefore, although nothing in the reaction mixture prevented the breakdown of cozymase, the dihydrocoenzyme II was stable.

Other N-alkyl heterocyclic compounds

Nicotinamide methosulphate and trigonelline (each 3×10^{-3} and $3 \times 10^{-4} \text{ M}$) did not lead to detectable nicotinamide, nicotinic acid or free acid when incubated with sheep or guinea pig brain preparations, reactions, 0.5% of the speed of that with cozymase, could have been detected. No acid was formed when the enzyme was incubated with benzimidazole methiodide or phenazine methosulphate at the same concentrations.

DISCUSSION

The major finding of the present experiments is that of the differential action of the coenzyme degrading enzyme on the oxidized, in distinction to the reduced, forms of the coenzymes. This is understandable when the structural differences between the two forms at the point of action of the enzyme are considered (Fig 1). The enzyme acts on a bond at an ionized quaternary nitrogen atom. In dihydrocozymase this is replaced by a tertiary nitrogen atom presumably with an excess instead of a deficit of electrons. As the breakdown of cozymase is little if at all affected by the presence of either of the dihydrocompounds, these appear to have little affinity for the enzyme. The minimum structure compatible with activity of the enzyme has not been defined by the present experiments, but would appear to be at least a nicotinamide nucleotide (as distinct from nucleoside). The action of the enzyme at varying stages of purification on coenzyme II, as well as on cozymase, renders inappropriate the terms 'diphosphopyridine nucleotidase' and 'diphosphopyridine nucleosidase' which have been applied to it. These names, also, do not discriminate between the present enzyme and the cozymase pyrophosphatase of Kornberg (1948). Substrate competition in our experiments also indicates that the degrading enzyme from the central nervous system acts on both coenzymes.

The coenzyme molecules contain some fifty bonds of which eight or nine are of types which are readily split by various enzymes, for example, the bonds between ribose and phosphoric acid or between the carbon and nitrogen of the amide. It is therefore noteworthy that the present enzyme attacks the point at which the coenzymes differ most from their dihydro derivatives.

The effects of this differential attack seem likely to be far reaching (McIlwain, 1949*a*, *b*). They can immediately be expected to include changes in redox

potential and in systems sensitive to such changes, and modifications of equilibria involving coenzyme catalysed dehydrogenases. Inhibition of cozymase breakdown by low concentrations of substances (including phenosafranine) which disturb the Pasteur effect (McIlwain, 1949*b*) suggests that the system degrading cozymase does indeed play a central part in carbohydrate metabolism in normal tissues, possibly participating in the phosphorylation associated with tissue oxidations (cf Judah & Williams-Ashman, 1949). It is noteworthy that in brain the speed of cozymase degradation was found (McIlwain & Rodnight, 1949) to be 2-3 times that of the oxygen uptake, both changes being expressed in molar quantities. The ratio appears to be similar in muscle and the enzyme exists in many animal tissues (Spaulding & Graham, 1947) although at a lower level.

SUMMARY

1 Preparations from the mammalian central nervous system, which rapidly inactivated cozymase, inactivated also coenzyme II at 70-85% of the rate of their reaction with cozymase.

2 Inactivation of coenzyme II, like that of cozymase, was accompanied by liberation of nicotinamide.

3 Competition took place between the two coenzymes for the degrading system, and presumably, therefore, a common enzyme was responsible for the two reactions.

4 Nicotinamide ribofuranoside, nicotinamide glycosides less closely related to cozymase, and several other nicotinamide derivatives were not affected by the enzyme.

5 Dihydrocoenzymes I and II were not affected by the enzyme, and did not affect the breakdown by it of coenzymes I and II.

We are greatly indebted to Mr J. C. Cheshire for assistance during these investigations.

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Apparent Vitamin C in the Walnut (*Juglans regia*)

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(Received 1 April 1949)

A previous communication (Wokes & Melville, 1948) described studies carried out over several years on the seasonal distribution of vitamin C in different tissues of the fruit and other parts of the walnut tree. The results showed that the translocation of vitamin C as such from the epicarp, leaf mesophyll and other photosynthetic centres, where the vitamin is probably synthesized, to the endocarp, where it occurs in such remarkably high concentration, seems to involve its passage up a steep concentration gradient presenting unusual difficulties as compared with the leaf to fruit gradients in most other species which have been investigated. In our studies on the walnut, apparent vitamin C was necessarily determined in all the samples, since the results for true vitamin C were determined generally by subtracting the figure for apparent vitamin C from that for total vitamin C. However, the data for apparent vitamin C were omitted from our previous paper in order to demonstrate the type of picture which is obtained if the presence and possible functions of the apparent vitamin C are ignored. The present paper supplies the missing data, enlarging and clarifying the picture.

Gerghelezhru (1937), who discovered the high concentration of vitamin C in unripe walnut fruits, did not seem to realize that his raw materials could contain considerable amounts of dye reductants which were not ascorbic acid, but this may have been due to his use of sulphur dioxide in the extracting fluid which, as will be shown later, can prevent the apparent vitamin C from reacting with the dye. His findings were confirmed by Pyke, Melville & Sarson (1942) and by Ranganathan (1942), but neither paper reported the presence of an appreciable amount of apparent vitamin C in the unripe walnuts examined, perhaps because these were too mature. Ranganathan (1942) stated that tests for interfering substances showed these to form 4–6 % of the total vitamin C. Taylor (1943) reported the presence of about 13 % of interfering substances in the total vitamin C in some unripe walnuts which he had examined. Melville, Wokes & Organ (1943) found the total vitamin C in six unripe fruits of *Juglans regia* and fifteen of allied species, to contain 12–73 % of interfering substances, for which they suggested the provisional term 'apparent vitamin C'. They used a modification of Lugg's (1942) formaldehyde

method (Wokes, Organ & Jacoby, 1943) in which the residual apparent vitamin C, after destruction of the true vitamin C by formaldehyde at pH 4.5, was titrated with the dye at a pH below 1.

Mapson (1943) found that some 14 % of the total dye titration for walnuts preserved in syrup was due to non specific reductants to which he applied the term 'reductones'. In his modification of Lugg's (1942) method, the 'reductones' were titrated at pH 0.6 and total vitamin C at pH about 1.2 (except when the presence of sulphides, sulphites or thiol compounds was suspected). His assumption that the compounds was suspected. His assumption that the apparent vitamin C in walnuts consisted of reductones was understandable in view of the finding of Wokes, Organ, Duncan & Jacoby (1943) that the apparent vitamin C in molasses, malt extract and certain fruit juices after prolonged storage closely resembled reductone or reductic acid in chemical and physical properties. Nevertheless, the apparent vitamin C in walnuts cannot be reductone, since its dye titration value is very low at pH 2 and increases markedly as the titration pH is lowered (Wokes, 1946*a*), in contrast with reductone, the dye titration value of which decreases as the titration pH is lowered (Martius & von Euler, 1934; Wokes, 1946*a*). The use of too high a titration pH probably explains the failure of Lugg & Weller (1943) and of Klose, Peat & Fevold (1948) to discover much apparent vitamin C in unripe walnuts, though it should be added that the latter workers complicated their results by using sulphur dioxide in the extracting fluid. On the other hand, when Tuba, Hunter & Osborne (1947) applied to walnuts Levy's (1943) method of estimating ascorbic acid in the presence of reductones and other interfering substances they found 24–38 % of the total vitamin C to be non specific dye reductants.

METHODS

Collection and dissection of material was as described previously (Wokes & Melville, 1948).

Estimation of apparent vitamin C

(a) *Formaldehyde method* The visual method described by Wokes, Organ & Jacoby (1943) was used, taking care to add after the formaldehyde treatment sufficient 25% (w/v) HPO_3 to ensure that the pH was brought down to 0.5–0.7

before titrating (This is the pH of the reaction mixture when determining total vitamin C) The pH of the titration mixture was determined and the result corrected to pH 0.7 by means of the curve previously published (Wokes, 1946a) When calculating from the dye readings the concentration of apparent vitamin C, its dye factor was assumed to be the same as that of ascorbic acid This procedure, the most convenient when estimating true vitamin C by difference, leaves the results for apparent vitamin C open to correction when its chemical nature is established It does not, however, affect their relative values When sufficient material was available, estimations of total and apparent vitamin C were occasionally made by the potentiometric method (Wokes, Organ & Jacoby, 1943) This always gave results in good agreement with the visual results, and also provided information on the effect of the titration time on the dye titration value (see p. 350) The fact, repeatedly confirmed, that the dye titration value increased as the pH was reduced below 1.5 gave strong reason for believing that the apparent vitamin C was not reductone

(b) *By using ascorbic acid oxidase* This enzyme, derived from cucumber juice, was found to destroy the whole of the ascorbic acid in walnut extracts without exerting any appreciable effect on the apparent vitamin C To ensure that the destruction of the ascorbic acid was complete, the mixture was incubated with the enzyme at pH 5 and at 38–40° until dye readings on samples became constant Recovery experiments carried out with different levels of added ascorbic acid gave similar results The data obtained in a typical experiment are plotted in Fig. 1 From the

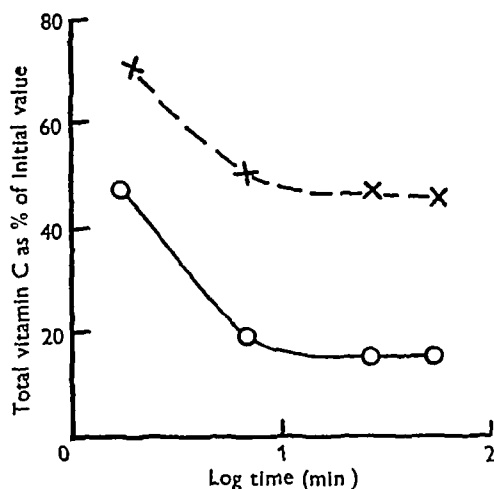


Fig. 1 Use of ascorbic acid oxidase to differentiate apparent from true vitamin C in walnut leaves. Results obtained on extract of leaves, \times — \times , results obtained on extract plus added ascorbic acid, \circ — \circ

general shape of the curves, it was clear that the enzyme had ceased to act on any dye reductant after about 20 min, either in the recovery or in the test experiment. The amount of apparent vitamin C then left in the two reaction mixtures was practically the same, indicating 349 and 344 mg/100 g respectively in the original leaves, as compared with about 320 mg/100 g by the formaldehyde method. Hence it was assumed that the enzyme had exerted no appreciable effect on the apparent vitamin C. Since ascorbic acid oxidase acts

slowly on reductone, further evidence was thus provided that the apparent vitamin C was not reductone. The method was not applied to many of the samples because it was tedious and required large amounts of material.

(c) *By differential titration* Since the dye titration value of the apparent vitamin C in walnuts falls to zero as the titration pH is raised above 2, it is possible to titrate the true vitamin C as such at a pH of about 3, then to add excess of HPO_3 to bring down the pH to 0.6 or 0.7 and continue the titration to determine the apparent vitamin C. In practice this method was found less convenient and accurate than the formaldehyde method, and was applied only on a few occasions, when it gave fair agreement with other methods.

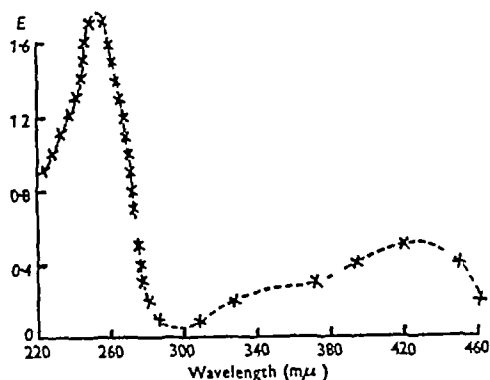


Fig. 2 Absorption curve of the yellow colour obtained when titrating walnut extracts with indophenol dye

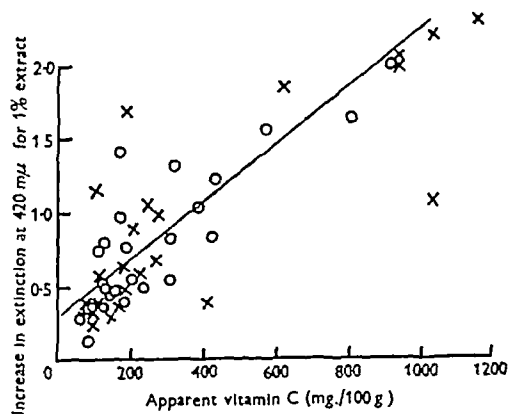


Fig. 3 Correlation of concentration of apparent vitamin C in walnut tissues with increase in yellow colour (measured spectrophotometrically at 420 mμ) when extracts of the tissues were titrated with indophenol dye. Results on fruit tissues indicated thus \times , results on stem and leaf indicated thus \circ . Regression line calculated from the equation $y = 0.0018 + 0.002047(x - 333)$

(d) *By increase in yellow colour* During titration of walnut extracts containing apparent vitamin C a yellow colour develops. Spectroscopic examination of this yellow colour revealed a broad absorption band in the visible spectrum with a maximum at about 420 mμ, and a narrow band of four or five times the intensity with a maximum at about 250 mμ (see Fig. 2). Using the Hilger Nutting spectrophotometer, we determined the increase in yellow colour at

420 mμ., caused by the dye titration, employing the cross over technique (Wokes & Still, 1942). The results, expressed as increase in $E_{1\text{cm}}^{1\%}$, plotted against the concentration of apparent vitamin C (in mg/100 g), determined by the formaldehyde method, are shown in Fig. 3, together with the regression line. The correlation coefficient for these data, $r=0.83$, was highly significant ($t=14.14$, $P<0.01$) and is strong evidence that apparent vitamin C usually consists of a single compound which is converted into a yellow pigment on oxidation. Nevertheless, occasional aberrant results preclude the use of this yellow colour for a routine estimation.

Loss of apparent vitamin C during dissection. In a number of experiments, the weights of apparent vitamin C in the dissected parts of leaflets and nuts were compared with the average yield from whole leaflets and nuts from the same spray. Losses due to dissection were found to be no greater than for true vitamin C in the same material (cf. Wokes & Melville, 1948). For example, in samples of leaves collected on 10 different days between May and September the weighted mean concentration of apparent vitamin C in dissected leaflets ranged from 85 to 136 and averaged 107% of the concentration found in whole leaflets ground without dissection, but in samples collected on 3 different days in October the corresponding percentages were 82, 71 and 44. Nuts were more variable, owing to a greater sampling error. For example, two dissected nuts gave 177 and 267 mg/100 g, averaging 222 mg/100 g, which was in reasonable agreement with the mean of 214 ± 23 mg/100 g obtained for ten whole nuts from the same sample, but the results on the individual nuts ranged from 131 to 382 mg/100 g. It is

probable that oxidizing enzymes capable of destroying apparent vitamin C are present in walnut tissues, since, on grinding with water and quartz sand, a yellow colour developed similar to that formed during titration. At the same time there was a rapid drop in the dye titration value. On the other hand, the apparent vitamin C in HPO_3 extracts of these tissues was fairly stable during storage in brown bottles under N_2 in the refrigerator, losses as low as 1–2% / month being frequently found after more than a year's storage, although considerably greater losses could occur if there was much exposure to air.

RESULTS

Whole fruits (nuts)

A seasonal trend in the concentration of apparent vitamin C in entire fruits is fully established, although the results are more variable than those previously reported for true vitamin C. Data on nearly 100 fruits from samples collected June–August 1944, May–August 1945 and June 1946, are summarized in Table 1. The highest concentration of apparent vitamin C was found in the youngest fruits weighing not more than 1 g, and was usually between 800 and 1000 mg/100 g. As the fruits grew, the concentration fell until at maturity it was only 0.2–0.1 of the initial value. The concentration of apparent vitamin C was therefore already at its height when the earliest samples were taken, whereas

Table 1. Seasonal changes in concentration of apparent vitamin C in walnut fruits

Sample no	Date gathered	Average wt of fruits (g)	Apparent vitamin C (mg /100 g)		Apparent vitamin C	
					(mg /fruit, mean)	(As % of total vitamin C)
			Individual fruits	Mean		
			From main tree			
1	7 June 1944	1	Pooled	800	8	62
2	13	1	Pooled	847	9	47
3	21	3	760, 715, 658	711	21	26
4	26	3.6	540, 520, 440, 430	483	17	19
5	4 July 1944	10	405, 325, 315	345	35	11
6	12	20.3	220, 275, 225, 320	260	51	14
7	18	17.5	185, 215, 120, 210	180	32	11
8	25	22.6	235, 195, 160, 248	210	48	13
9	1 Aug 1944	24.9	155, 150, 120, 115	135	36*	11
10	9	32.1	135, 148	141	45*	14
11	22	21.7	190, 140	165	36	12
25	17 May 1945	0.3	Pooled	789	2.4	46
26	30	0.5	716, 475, 539	577	3	35
27	6 June 1945	1.75	568, 501	535	9	23
28	12	2.8	401, 323, 380	368	10	16
29	19	4.1	429, 308, 384	348	14	17
30	26	14.0	243, 205, 234	227	32	14
31	10 July 1945	25.2	197, 168, 139	168	42	15
32	23	29.7	158, 174, 172	168	50	14
33	8 Aug 1945	32.0	87, 100, 103	97	31	9
45	18 June 1946	0.3	Pooled	525	1.5	43
From other trees						
242	29 June 1944	10.6	455, 405, 390	417	44	17
243	25 June 1945	18.5	223, 140, 200, 214, 194, 167	190	35	10

* Excluding woody portion

the peak value was not reached for true vitamin C until about a fortnight later and then fell gradually as lignification of the endocarp proceeded. The occurrence of peak values for these substances at different times at first suggested that apparent vitamin C might be a precursor of ascorbic acid in the plant. This idea had to be abandoned later when evidence was found that apparent vitamin C is related to juglone. The total amount of apparent vitamin C in a single fruit increases during growth from about 2 mg to 50 mg, whereas true vitamin C increases much more rapidly from about 3 mg to over 300 mg per fruit. Thus, there is finally only about one sixth as much apparent as true vitamin C. The change in proportion does not suggest any close physiological link between these substances in the plant, and no correlation was found between the observations (1944) of the true and apparent vitamin C in entire fruits. There was, however, a high correlation ($r=0.99$) between apparent vitamin C and true vitamin C a fortnight later ($t=28.37$, $P<0.01$). This result may be due to parallel trends in unrelated processes rather than to a causal relation.

Separate tissues of the fruits

Fruits were dissected as previously described and the separated epicarp, mesocarp, endocarp and kernel were assayed for both true and apparent vitamin C, the same fruits providing data for the whole fruit and separate tissues.

Initial high concentrations (well over 1 g/100 g) of apparent vitamin C were found in the epicarp and mesocarp (Table 2). These values declined as growth proceeded, but there was a final small rise in the full grown fruits. The concentrations in the endocarp were much lower, but in general showed similar trends. A definite concentration gradient was generally demonstrable from the epicarp and mesocarp towards the endocarp, and there was always a clear gradient into the testa and its contained endosperm or embryo. Often no positive gradient could be shown from the entire fruit stalk into the endocarp, but, in contrast with true vitamin C, a gradient through the phloem could usually be established as the concentration of apparent vitamin C was higher than in the other tissues. The final rise in concentration in the endocarp was greater if the non-lignified portion alone was considered. This could be explained by a differential partition of the apparent vitamin C at a stage when there was little difference in concentration between epicarp, mesocarp and endocarp. Correlations between true and apparent vitamin C in the fruit tissues were investigated, but the only significant correlation found was negative, between true and apparent in the epicarp ($r=-0.93$, $t=5.02$, $P<0.01$). At first this was interpreted as evidence that apparent vitamin C was a precursor of ascorbic acid, but later work, indicating a relationship to juglone, appears to rule out that view. The same difficulty arises with

Table 2 *Seasonal distribution of apparent vitamin C in different tissues of walnut fruits*

(Dates of collection of samples are given in Tables 4 and 7)

Sample no	Fruit stalk	Apparent vitamin C (mg/100 g)				
		Epicarp	Mesocarp	Endocarp	Testa	Endosperm
1	229	1616	—	92	—	—
2	—	1497	—	203	—	—
3	—	712	924	102	—	—
4	120	595	1368	185	—	—
5	145	405	610	65	20	—
6	—	195	265	150	5	1
7	—	125	275	125	5	1
8	—	150	240	110	5	1
9	200	120	200	100*	9	1
10	120	130	145	160*	27	1
11	125	240	320	210*	12	1
26	106	1029	623	112	—	—
27	95	716	1094	117	—	—
28	106	383	802	111	—	—
29	81	428	919	134	—	—
30	63	145	411	82	—	—
31	134	102	235	139	—	—
32	108	185	122	129	3†	
33	88	69	112	204	—	—
34	30	167	229	—	—	—
2k1	—	450	780	78	—	—
2k2	—	265	715	30	10†	
2k3	—	215	361	66	8†	

* Excluding woody portion of endocarp

† Result on whole kernel

the correlation ($r=0.87$) found between apparent vitamin C in the epicarp and true in the endocarp a fortnight later ($t=4.85$, $P<0.01$)

Leaves

Data on leaves are given in Table 3. Results on twenty one samples show that the concentration of apparent vitamin C was always highest in the mesophyll, where there was no definite seasonal change, whereas the concentration of true vitamin C fell gradually with age. While the fruits were growing, there was a clear concentration gradient of apparent vitamin C from mesophyll through the leaf vascular tissue and rachis as far as the leaf base. Hence a gradient into the main stem was more difficult to establish, especially from older leaves, despite attempts to allow for the proportion of phloem in the tissue examined.

apparent vitamin C in the vegetative buds usually increased in the early summer, shortly before they opened, but fell again rapidly as the tiny leaves grew. When the male catkin buds were just distinguishable in late summer, the concentration of 2-3 g/100 g was higher than in any other plant tissue, but fell slowly during the autumn. Simultaneously, their average weight increased from about 50 to 80 mg, so that the amount of apparent vitamin C in a catkin bud remained at about 1 mg. In the spring, the male catkins grew rapidly till they weighed more than 1 g each. The concentration of apparent vitamin C fell steadily to less than 500 mg/100 g. The amount in an average catkin fell slightly at the beginning of the spring growth, but then increased to 3 or 4 mg. As the catkins now contained a good deal of fresh green tissue, it is possible that some of this apparent vitamin C was formed by

Table 3 *Apparent vitamin C in walnut leaves*

(Dates of collection of samples are given in Tables 4 and 7)

Sample no	Apparent vitamin C (mg/100 g)					
	Whole leaflets	Mesophyll	Vascular tissue	Rachis	Petiole	Main stem, cortex and phloem
9	250	—	—	—	120	—
11	—	340	160	—	120	—
25	243	299	233	260	119	91
26	242	231	150	141	94	132
27	215	206	182	116	87	173
28	243	277	171	95	95	151
29	271	411	182	109	106	259
30	144	169	95	91	48	304
31	204	349	116	83	81	245
32	192	226	94	94	72	459
33	224	325	118	106	87	536
34	427	410	310	158	51	546
35	243	—	—	37	36	97
36	360	298	291	52	76	179
37	234	68	116	116	68	156
38	202	235	117	64	36	500
43	306	—	—	—	—	412
44	270	542	363	235	—	232
45	195	203	199	162	136	123
46	192	211	170	111	131	169

Buds, catkins and main stem

Data obtained from August 1944 to October 1946 are given in Table 4. The concentration of apparent vitamin C in the buds often exceeded 1 g/100 g, but varied widely from time to time. This was not entirely due to the sampling error, since the results on vegetative buds were usually similar to those on reproductive buds in the same sample. In the early summer, when the reproductive buds began to develop into female catkins, there was a considerable increase in their size so that, although the total amount of apparent vitamin C in an average bud had increased, its concentration remained steady or even fell. On the other hand, the concentration of

photosynthetic activity in the catkins, and was not translocated from other parts of the tree. Since the concentration in the adjacent stem, cortex and phloem at this time was 300-500 mg/100 g, and about 5% of this consisted of sieve tissue, a comparatively small differential concentration in the phloem would have ensured a positive translocation gradient into the catkins. At other times of the year, the observed concentrations were less favourable to direct translocation into any type of bud, though it is not impossible that a positive gradient always existed in the phloem serving these buds. The data on the cortex and phloem from different parts of the stem showed that from October to April the concentration gradient of apparent vitamin C was con-

Table 4 *Seasonal distribution of apparent vitamin C in buds, catkins and stem*

(C Y G = Current year's growth P Y G = Previous year's growth)

Apparent vitamin C (mg /100 g)

Sample no	Date gathered	Stem (cortex and phloem)			Buds		Male catkins	Apparent vitamin C (mg /catkin)
		C Y G	P Y G	Older	Vegetative	Reproductive		
12	29 Aug 1944	215	—	—	—	995	2530	1.3
13	4 Sept 1944	—	—	—	988	1040	2430	1.3
14	11	—	—	—	970	813	—	—
15	10 Oct 1944	157	—	—	728	785	1485	1.1
16	14 Nov 1944	795	—	—	1200	1040	2690	1.6
17	20	580	—	—	1220	—	2310	1.5
18	27	335	—	—	1080	1180	2130	1.3
19	8 Jan 1945	340	—	418, 369	711	—	1030	0.8
20	21	297	195	119, 156	472	604	1467	0.9
21	28 Feb 1945	310	264	174, 61	435	273	1146	0.9
22	13 Mar 1945	444	202	201	627	575	762	0.9
23	4 Apr 1945	306	—	—	371	568	481	3.4
24	13	—	—	—	—	—	488	4.3
25	17 May 1945	91	369	—	1022	776*	—	—
26	30	132	143	—	1190	577*	—	—
27	6 June 1945	173	409	523, 502	1132	—	—	—
28	12	151	360	—	935	615	—	—
29	19	259	287	434	1033	—	—	—
30	26	304	424	—	1033	—	—	—
31	10 July 1945	245	309	—	936	—	—	—
32	23	459	481	—	951	—	—	—
33	8 Aug 1945	536	576	—	1051	—	—	—
34	11 Sept 1945	546	446	340, 382	1214	—	2592	—
35	2 Oct 1945	97	187	130, 87, 50, 23	964	1045	1919	—
36	9	179	180	183	292	—	1041	0.7
37	15	156	149	—	454	—	1303	0.9
38	22	500	156	—	695	—	1878	1.4
39	31	323	176	—	616	—	1674	1.2
40	13 Nov 1945	269	201	—	817	—	1617	1.1
41	15 Jan 1946	291	212	—	769	—	1535	0.9
B	21 Mar 1946	252	—	—	—	—	—	—
42	2 Apr 1946	189	119	—	499	500	699	1.4
43	15	412	223	—	412	—	354	2.9
44	29	235	259	—	581	—	198	—
45	18 June 1946	123	388	—	—	—	—	—
46	1 July 1946	169	303	—	1043	—	—	—
47	23 Sept 1946	—	—	—	—	—	2200	0.6
48	24 Oct 1946	100	179	248	365	—	1200	0.9

* Excluding woody portion

sistently from new to older tissue and could be traced some considerable distance down the stem. From May to September the observed concentration gradient was consistently in the reverse direction, from older to newer tissue. In March 1945 we estimated the apparent vitamin C in all parts of a young walnut sapling. Our results (see Table 5) showed a concentration gradient from the cortex plus phloem of the previous year's growth down into the fine roots, where, however, there was still about 300 mg /100 g, several times the concentration of true vitamin C. The concentration in the cortex plus phloem of the current year's growth was lower than in the previous year's growth, but this may have been due to a higher proportion of cortex in the former. The value for the previous year's growth was

Table 5 *Apparent vitamin C in walnut sapling*

	Apparent vitamin C	
	(mg /100 g)	(As % of total)
Buds	596	70
Stem (cortex plus phloem)		
Current year's growth	298	54
Previous year's growth	415	69
Roots	327	71
Fine roots	302	76

checked by a control experiment with added ascorbic acid on a second sample. The result (386 mg /100 g) was in fair agreement with the previous result of 415 mg /100 g and showed that the whole of the true vitamin C was being destroyed by the formal dehyde under the experimental conditions.

Storage experiments

From the evidence of concentration gradients apparent vitamin C seemed much more likely than true vitamin C to be translocated from leaf to fruit during the growth of the latter. This led to the hypothesis that the apparent vitamin C, after its arrival in the endocarp, may yield true vitamin C

vitamin C in the fruits dissected was about 20% lower than that in whole fruits. In 1945, a further storage experiment was made, extending over a period of 2 months (Table 7). The results, corrected for changes in weight during storage, showed no significant changes in the concentrations of either true or apparent vitamin C during the period, thus corroborating the experiment of 1944.

Table 6 *Changes in average concentration of true and of apparent vitamin C in walnut fruits during storage*

(Storage for 16 days at 4–5° in July 1944, all results given as mg/100 g)

	Whole fruit		Epicarp		Mesocarp		Endocarp		Kernel	
	True	Apparent	True	Apparent	True	Apparent	True	Apparent	True	Apparent
After storage	2337	379	1025	233	963	704	3046	58	35	2
Before storage	2035	417	1195	265	1085	715	3140	30	140	10
Increase or decrease during storage	+302	-38	-170	-32	-122	-11	-94	+28	-105	-8
S.D. of mean (after storage)	171	37	17	29	31	61	4	10	—	—
S.D. of mean (before storage)	11	20	—	—	—	—	—	—	—	—

for which it has served either as a precursor or as a carrier. In order to test this hypothesis, we carried out in 1944 a storage experiment with some immature walnuts which, after sampling for assay, were stored in the refrigerator at 4–5° in a box with access to air. The results (Table 6) showed, after 16 days' storage, an increase of 302 mg/100 g in the concentration of true vitamin C and a decrease of 38 mg/100 g in the concentration of apparent vitamin C. (Allowance is made in these figures for the gradual loss in weight of the fruits during

Extraction experiments

Apparent vitamin C was found to be readily soluble in ethanol and aqueous solvents, but much less soluble in solvents immiscible with water, such as benzene, light petroleum, methyl or ethyl ether, chloroform, isobutanol, carbon tetrachloride, ethyl acetate and carbon disulphide. Thus, purification by phase separation seemed unpromising. Whilst it could be extracted from fresh walnut tissues by grinding with ethanol and quartz sand, this often led to heavy losses which could be avoided by adding a small amount of hydrochloric acid to the solvent (2–5% of concentrated acid by volume). The extracts contained much chlorophyll, which rendered visual dye titrations difficult. The chlorophyll could be removed by shaking with light petroleum, leaving a pinkish yellow solution of apparent vitamin C in the acid ethanol. The acid could be removed by evaporation *in vacuo*, yielding very unstable residues with initial concentrations of apparent vitamin C as high as 7%.

Rate of reaction with indophenol dye

One of the most characteristic properties of apparent vitamin C is its slower rate of reaction with the dye, compared with the almost instantaneous reaction of the latter with ascorbic acid. The slower rate can be detected in visual titrations, but is particularly well marked in potentiometric estimations. Wokes, Organ & Jacoby (1943), using a modification of the potentiometric method of Harris, Mapson & Wang (1942), showed that the amount of apparent vitamin C found in a stored sample of dehydrated carrot could vary from 20 to 90 mg/100 g as the time taken to carry out a titration was

Table 7 *Average concentrations of true and of apparent vitamin C in whole walnut fruits at intervals during storage*

(Storage 4–5° in 1945, all results given as mean \pm S.D.)

Date examined	Period of storage (days)	No. of fruits examined	Vitamin C (mg/100 g)	
			True	Apparent
29 June	0	6	1849 \pm 57	193 \pm 15
5 July	6	4	1910 \pm 138	243 \pm 51
13	14	4	1363 \pm 159	196 \pm 28
19	20	4	1869 \pm 37	238 \pm 15
7 Aug	39	4	1798 \pm 90	198 \pm 33
27	59	4	1815 \pm 38	180 \pm 30

storage). Unfortunately, owing to the large sampling error, these differences did not reach significance. Moreover, the loss of apparent vitamin C was very much smaller than the gain of true vitamin C. Results on dissected tissues were also inconclusive, the data indicating a greater loss of true than of apparent vitamin C throughout. Ascorbic acid oxidase liberated during dissection may have caused this loss as the tissues had shrunk and become more brittle during storage. This view was supported by the observation that the mean concentration of true

increased from about 2 to 30 min. This observation justifies the importance we have attached to the control of the titration time. Attempts have been made to estimate true vitamin C in the presence of these interfering substances by adding a known excess of dye and measuring photoelectrically at short intervals (e.g. 15, 30, 60 sec. after the dye addition) the amount of unreduced dye. The readings are plotted against time and extrapolated back to zero time to estimate the true vitamin C. The interfering substance could then, if desired, be estimated by difference, assuming that the reaction

spectively about half and a fiftieth of that for ascorbic acid, and utilized these in a method of estimating ascorbic acid in the presence of reductones which gave good agreement with the formaldehyde method. Their method would not, however, distinguish between ascorbic acid and *iso*ascorbic acid or hydroxytetronic acid, because the velocity constants of these acids with the dye at pH 1.4 and at 3.5 were too close to one another. Such differentiation could be effected by the infrared absorption method of Trotter, Thompson & Wokes (1948), but this method was not available for the work described

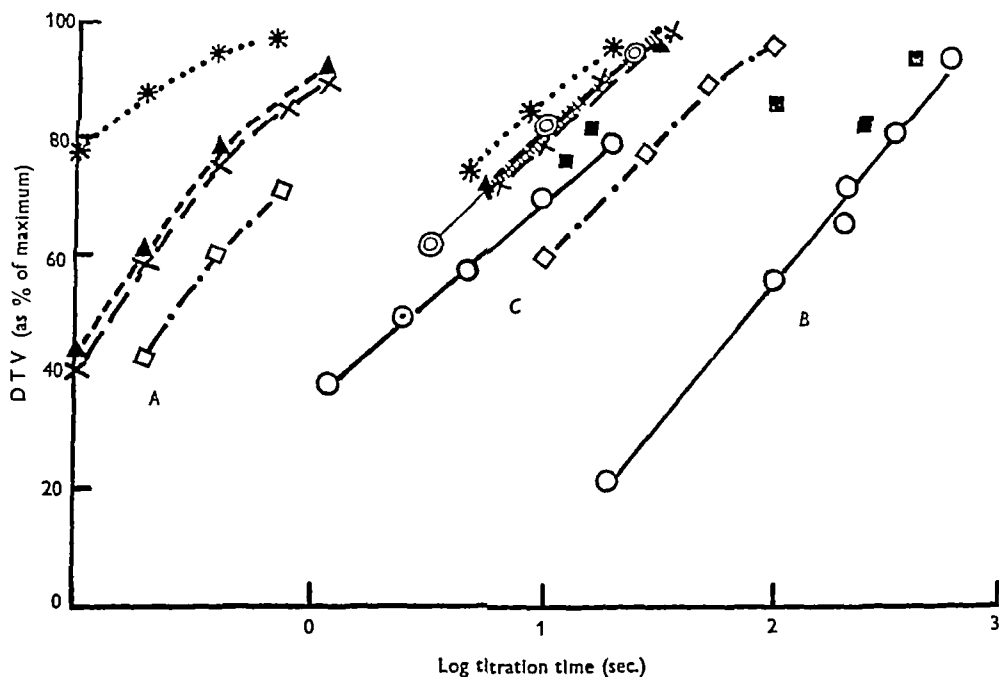


Fig. 4. Effect of titration time on dye titration value (DTV) of ascorbic acid and allied compounds. Ascorbic acid, \times — \times , hydroxytetronic acid, \blacktriangle — \blacktriangle , dihydroxy maleic acid, $* \cdot \cdot *$, reductic acid, \diamond — \diamond , glucoreductone, \circ — \circ , diketogulonic acid, \odot — \odot , *iso*ascorbic acid, \bullet |||| \bullet . Results obtained by Harris & Mapson (1947) on left-hand side (A), by Wokes (1946b) on right-hand side of diagram (B). Results of present work in centre (C). Results on walnut extracts indicated thus \blacksquare .

time was adequate. This method certainly corrects for some of the interfering substance, but the correction is not necessarily complete as, in the short but definite time required for the dye to react with the whole of the ascorbic acid, it may also have acted with some of the interfering substance. This was clearly shown by Harris & Mapson (1944, 1947) using the continuous flow method, with which they used reaction times down to 0.28 sec. They found that at pH 1.4 about 12% of ascorbic acid had not yet reacted with the dye 1.1 sec. after mixing, whereas about 40% of reductone had already reacted in this time under the same experimental conditions. They obtained velocity constants for reductic acid and for reductone which were re-

in the present paper. However, no indication has been obtained of the presence of *iso*ascorbic acid or of hydroxytetronic acid in walnut tissues. Wokes (1946b) described a modified potentiometric method with which reaction times were obtained to less than 10 sec. Using a titration pH of 0.6–0.7, he confirmed that *iso*ascorbic and hydroxytetronic acids reacted as quickly as ascorbic acid with the dye, and that dihydroxymaleic acid reacted still more rapidly. He also found that under the given conditions diketogulonic acid reacted as rapidly as ascorbic acid, reductic acid rather more slowly, and reductone much more slowly (see Fig. 4). Apparent vitamin C from different walnut tissues resembled reductone in reacting more slowly.

Spectroscopic studies

For these we used purified acid ethanolic extracts prepared, as described above, from different walnut tissues, but especially the buds and catkins, in which the dye titrations had shown the apparent vitamin C to represent practically the whole of the dye reductant. The extracts were examined using the

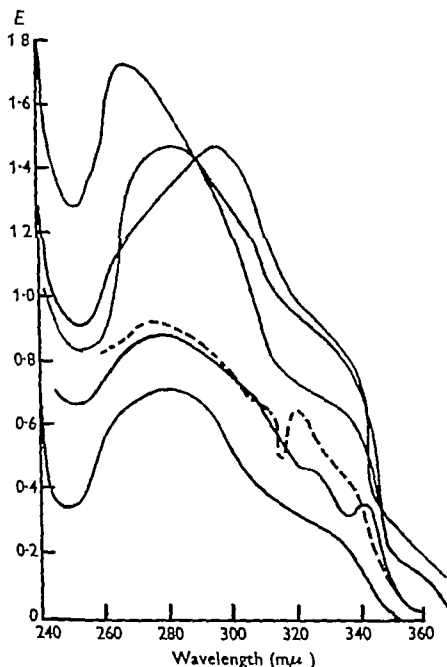


Fig 5 Absorption curves of extracts of walnut buds and catkins, all in HPO_3 , except the dotted curve which was obtained on an extract the pH of which had been adjusted to 4.5

Hilger E3 quartz spectrograph, and showed a broad absorption band with a main peak at 280–295 and indications of subsidiary peaks at 325–330 and 335–343 $\text{m}\mu$ (Fig 5). Except in one rather doubtful case, we were unable to obtain any evidence of the presence of reductone or of reductic acid, two of the possible constituents of apparent vitamin C which have been found in other plants.

DISCUSSION

The discovery in walnut tissues of extraordinarily high concentrations of apparent vitamin C raised interesting questions regarding its nature and possible functions. In earlier work we were guided by the hypothesis that the apparent vitamin C resembled that found in other foods and was probably an enediol of the reductone type. This hypo-

thesis was supported by a close resemblance to reductone in its slow rate of reaction with the dye, a criterion which Harris & Mapson (1947) used to differentiate between various non specific dye reductants. However, its failure to react with ascorbic acid oxidase, under conditions such that reductone would react, led us to doubt this hypothesis, and finally to abandon it on discovering (Wokes, 1946a) that, in contrast with reductone, the apparent vitamin C of walnuts showed a marked increase in dye titration value as the pH was reduced below 2. This was confirmed by our failure to detect reductone spectroscopically in numerous extracts of different walnut tissues, especially buds and catkins. The observation that a yellow colour developed when the apparent vitamin C was titrated with dye, or oxidized with the walnut enzymes, led us to investigate this oxidation product, and when we found that it had a similar spectrum to that of juglone, the possibility arose that some at least of the apparent vitamin C in walnuts is a reduced form of juglone. Although there was a high correlation between the increase in intensity of yellow colour and the concentration of apparent vitamin C in different tissues, occasional discrepant results suggested that in some instances at least there might be a second non specific dye reductant present. Spectroscopic studies of numerous walnut extracts failed to identify the apparent vitamin C, the absorption curves showing evidence of fine structure which needed for its complete elucidation more accurate and more sensitive spectroscopic apparatus than was at our disposal.

The occurrence of apparent vitamin C in so many of the walnut tissues, especially those in which ascorbic acid is also found, suggests that it cannot be merely a waste product, but almost certainly plays an active part in some metabolic process, possibly one concerned with ascorbic acid. Had it proved to be reductone, as earlier postulated, it might then have been a precursor of ascorbic acid. Since reductone has been formed *in vitro* by the action of ultraviolet light on glyoxal, which is itself a possible product of photosynthesis, an attractive hypothesis could be developed for the photosynthesis of ascorbic acid from glyoxal via reductone. Probably in most plants synthesis of ascorbic acid occurs in green photosynthetic tissues, to be followed by translocation to the fruits. This could explain our failure to find apparent vitamin C in fruits or leaves of other plants. In the walnut, synthesis of ascorbic acid in the leaves might be incomplete, some of the reductone being translocated as such to the endocarp there to be converted into ascorbic acid. Such a picture would fit many observations, especially those concerning concentration gradients, which are more favourable to the translocation of apparent than of true vitamin C from leaf to fruit.

The above theory was discredited by our failure to obtain a significant increase in vitamin C concentration during the storage of immature fruits in which the concentration of apparent vitamin C was falling. It was abandoned when we obtained clear evidence that the apparent vitamin C is not reductone, but is more likely to be a juglone derivative, possibly a reduction product. Such a compound could hardly be a precursor of ascorbic acid, though it might be linked with the latter in an oxidation system. Further speculation must, however, be deferred until the chemical nature of apparent vitamin C has been more clearly established. Work on this problem is still in progress.

Since the above work was completed evidence has been obtained (Daglish & Wokes, 1948) that the apparent vitamin C in resting buds and catkins is mainly, if not entirely, a derivative of hydrojuglone (1,4,5-trihydroxynaphthalene).

SUMMARY

1 Apparent vitamin C has been detected in numerous samples from the walnut (*Juglans regia*).

2 In fruits the highest concentrations occurred in the epicarp and mesocarp, especially in very young fruits, in which it could form half of the total vitamin C, as measured by dye titration.

3 In leaves the highest concentrations occurred in the mesophyll, from which there was usually a definite concentration gradient through the vascular tissue, rachis and petiole towards the stem.

4 In the stem the highest concentration was in the phloem, in which the concentration gradient was generally from older to younger tissue during the early summer, but in the reverse direction in the winter.

5 Remarkably high concentrations of apparent vitamin C were found in buds and catkins, in which it usually formed most of the total vitamin C.

6 The rate of reaction of apparent vitamin C with the indophenol dye was found to be much slower than that of ascorbic acid, dihydroxymaleic acid, diketogulonic acid, hydroxytetronic acid or isoascorbic acid, and to lie between those of reductic acid and reductone, all determined at the normal titration pH of about 0.7.

7 The apparent vitamin C in walnut tissues differs from reductone in (a) its stability towards ascorbic acid oxidase, (b) the increase of its dye titration value as the titration pH is reduced below 2, (c) the development, by the action of the indophenol dye or of the walnut enzymes, of a yellow compound which spectroscopically seemed to resemble juglone.

We are indebted to Prof. T. Reichstein for specimens of reductone and of reductic acid, to Dr F. Bergel for specimens of reductone, reductic acid, diketogulonic acid, hydroxytetronic acid and isoascorbic acid, to the late Dr F. C. Jacoby for many of the spectroscopic estimations and to Miss J. G. Organ, Miss E. M. James and Miss E. Finnegan for assistance.

Note. Since this paper was sent to the press we have been shown by Prof. Mirmanoff a thesis for Doctor of Pharmacy dealing with vitamin C in the walnut and confirming some of our findings. This thesis (Stadelmann, 1949) refers to work carried out in Germany during the war by J. Breinlich (Breinlich, 1942), in which the suggestion is put forward that the ascorbic acid in walnuts is oxidised to dehydroascorbic acid by juglone, which is itself reduced to hydrojuglone. No proof is provided of the existence of hydrojuglone in walnut tissues.

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Chemical Factors in the Germination of Spore-bearing Aerobes. The Effect of Yeast Extract on the Germination of *Bacillus anthracis* and its Replacement by Adenosine

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(Received 29 March 1949)

The rapidity of germination of bacterial spores when inoculated into a nutrient medium has been known for many years. Thus Fischeoeder (1909) showed that 98% of anthrax spores became heat sensitive in broth at 37° in 1 hr, but only 50% became heat-sensitive in saline at the same temperature in 5 hr. Little work has been done, however, on the specific nutrients responsible for this type of effect, in spite of advances in the study of the requirements for bacterial growth (reviewed by Knight, 1945). Thus the review of Knaysi (1948) on the bacterial endospore adds little on the conditions for germination to that of Cook (1932), apart from the work of Knaysi (1945) himself on *Bacillus mycoides* (*B. cereus* var. *mycoides*). Using a microscopic technique, he concluded that the spore is adequately supplied with stored nitrogen, but requires a source of carbohydrate and maintenance of a neutral pH. On the other hand, Keilin & Hartree (1947), using a metabolic method, found that *B. subtilis* required a source of assimilable nitrogen and an unidentified dialysable factor present in yeast extract, peptone or tryptic digest of casein.

More recently, Wynne & Foster (1948*a, b, c*), and Foster & Wynne (1948), have studied the subject with special reference to *Clostridium botulinum*. Their findings showed a need for carbon dioxide (Wynne & Foster, 1948*c*), and inhibition by traces of unsaturated fatty acids, recalling inhibition of the growth of non-spore-forming bacteria, e.g. *Mycobacterium tuberculosis* (Dubos, 1945) and *Haemophilus pertussis* (Pollock, 1947). In spite of the widespread occurrence of carbon dioxide requirements and unsaturated fatty acid inhibitions in bacterial nutrition, no such effects were observed (Wynne & Foster, 1948*c*, Foster & Wynne, 1948) with the germination of the four aerobes studied, *B. brevis*, *B. megatherium*, *B. mesentericus* (*B. pumilus*) and *B. subtilis*.

In other fields of biology, there has been a general interest in problems of dormancy, and it has been recognized that the germination of many dormant cells is stimulated by essential metabolites produced and secreted by active tissues (see Brown, 1946), and in some instances the active principles have been either identified chemically or replaced by pure chemical compounds.

The germination of the mould, *Phycomyces blakesleeana*, was examined by Robbins and co-workers, who obtained two fractions from potato extract (Robbins & Hamner, 1940), both of which were essential for optimal activity. One of them could be replaced by guanine (Robbins & Kavanagh, 1942*a*) or by hypoxanthine (Robbins, Kavanagh & Kavanagh, 1942), and the latter was isolated from the extract by Robbins & Kavanagh (1942*b*). Other purines tested by Robbins (1943) were less active. The work of Hurni (1946) showed that the germination of *Rhizopus nodosus* was also stimulated by hypoxanthine, and that the early growth of four other Mucoraceae was similarly stimulated, though proof was not given that such stimulation was specifically connected with the phase of germination. With *Neurospora crassa*, on the other hand, the stimulating material in media containing autoclaved pentose was probably furfural (Emerson, 1948).

In a different field, the requirements for excystment of the ciliate protozoon, *Colpoda cucullus*, have been reported by Barker & Taylor (1933), Thumann & Barker (1934), Thumann & Haagen Smit (1937), Haagen Smit & Thumann (1938) and Prater & Haagen Smit (1940). These authors found that, in addition to a source of soluble carbohydrate and various stimulating factors associated with carbohydrate metabolism, there was a need for one of two neutral compounds, $C_{13}H_{24}O_2$ or $C_{26}H_{44}O_2$, which were active at a concentration of 1 in 2.5 – 5.0×10^7 . So far, it is not known if the nature of these compounds has been established, and their activity proved by the use of synthetic specimens.

It will be seen that in addition to unidentified factors, the compounds which have been implicated in the germination of dormant cells are of a very varied nature, no common pattern being yet discernible. The work now reported is concerned with the addition of results on the germination of spores of a single strain of *B. anthracis*. Results on other strains and species will be given in a later paper.

METHODS

The loss of resistance to heat, as a physiological criterion of germination shown by viable count, has been preferred in spite of the criticism (Cook, 1932,

Gardner, 1945) that this represents a pregerminative phase and does not correspond to true germination. The choice is justified, however, by the customary use of heat resistance in the diagnosis of spore formation and in freeing spore suspensions from vegetative cells. Support of this view has recently been elaborated by Wynne & Foster (1948a), and the evidence need not be repeated in detail. Briefly an organism, after loss of heat resistance, cannot be regarded as being in the spore state. A spore undergoing such a change must therefore be regarded as having germinated if still viable. The term 'germination' will therefore be used in this sense throughout.

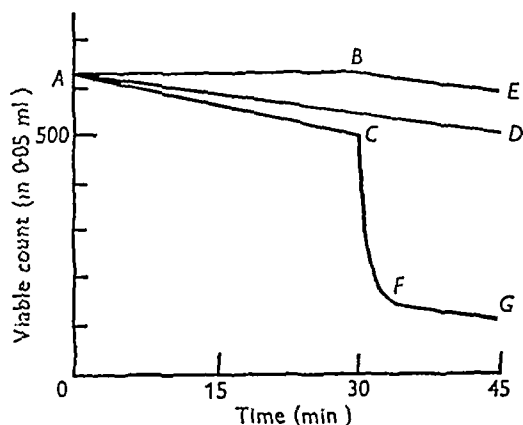


Fig. 1. Effect of time at different temperatures on viable count of *Bacillus anthracis* (diagrammatic). *AB* at 0° in nutrient medium, *AC* at 35° in nutrient medium, *AD*, *BE* and *CFG* at 60° in inert diluent.

General principle. This is shown in Fig. 1. If spores were suspended in a suitable culture medium, and dilutions in a non-nutrient but harmless fluid were made at intervals for determination of the viable count, the changes in count were either small or insignificant during a period of about 30 min. whether the culture medium was maintained at 0° (*AB*) or 35° (*AC*). If the dilutions were heated at 60°, little further change was shown over a period up to 60 min, either with those made initially (*AD*) or with those made from culture medium which had been maintained at 0° (*BE*). With dilutions from culture medium which had been maintained at 35°, on the other hand, the count fell rapidly (*CFG*), usually reaching a comparatively stable lower level in less than 5 min. The count of spores remaining resistant to heat at 60° after any treatment will be referred to as the *S* count, while counts set up without heating at 60°, including both resistant spores and non-resistant vegetative forms, will be referred to as the *S* + *V* count. (These abbreviations are used instead of the terms 'spore count' and 'total count' to avoid confusion with the customary meaning of the latter in bacteriology.)

Spore suspensions. Spores of a virulent strain of *B*

anthracis, supplied by Dr R. L. Vollum, were dried in horse serum and stored *in vacuo*. Three different spore suspensions were prepared from this stock culture as follows: (a) Grown direct from the stock culture on the surface of CCY agar (Gladstone & Fildes, 1940) to about 50% sporulation in 2-3 days; (b) Grown from (a) to about 100% sporulation in 24 hr in a vigorously aerated medium composed of 2% marmite, 1% cane molasses (decolorized with activated charcoal) and 0.5% $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$. The pH of this acid medium was adjusted to 7.3 with aseptic precautions after autoclaving; (c) Grown from (a) to about 90% sporulation in 48 hr in the 'complete' synthetic medium of Gladstone (1939) with L-alanine in place of DL-alanine (Hills, 1949) and supplemented by 10^{-7}M aneurin (Hagan, O'Kane & Young, 1943), $5 \times 10^{-4}\text{M}$ Ca^{++} (Brewer, McCullough, Mills, Roessler, Herbst & Howe, 1946), and 10^{-5}M yeast adenylic acid. The inoculum was about 2000 spores/ml (viable count), and the cultures were aerated by shaking at 120 oscillations/min in 50 ml conical flasks containing 10 ml culture, the distance of travel being 5 cm. The strain was still virulent after growth on this synthetic medium (LD_{50} for mice, about 200 spores by the intraperitoneal route).

After reaping, all spore suspensions were heated 1 hr at 60° to destroy remaining vegetative forms, washed thrice with distilled water and stored in the ice chest. Suspension (a) was used for the major part of the work, (b) was used only for a few preliminary experiments, and (c) was used after the discovery that a preparation of yeast adenylic acid promoted almost complete germination in a suitable synthetic medium. It was thus possible, by cultivation of spores in such a medium, to reduce transference with the inoculum of unknown biologically active metabolites. Without adenylic acid, the use of a synthetic medium involved cultures in which only a small proportion of the inoculum could have been shown to have germinated. Under these conditions, growth from those spores which germinated most easily may have produced a new population of spores with simpler requirements for germination than those of the original culture. Such suspensions were avoided.

Basal medium. One of the early difficulties was the design of a suitable deficient basal medium, since germination was too rapid for quantitative measurement in the usual laboratory media.

The medium finally adopted had the following composition: KH_2PO_4 (A.R., 4.5 g in 450 ml water), L-tyrosine (crude, see p. 357, 50 mg in 25 ml N NaOH), HCl hydrolysed gelatin (Nelson's photographic no. 1, neutralized, 12.5 ml 20%). The constituents were made up together to 500 ml, brought to pH 7.4 with 1-2 ml N NaOH and autoclaved. This basal medium was double strength to allow for the addition of inoculum and various test preparations to a fixed total volume. Although not essential for germination, the following mixture, which was essential for good growth, was added aseptically just before use (0.5 ml/10 ml double strength medium): aneurin, 1 ml 10^{-4}M ; MgSO_4 , 40 mg; $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 16 mg; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 110 mg; glucose, 2.5 g, in 5 ml N HCl with 20 ml water, Seltz filtered. After this addition the medium was neutralized with 0.5 N NaHCO_3 (0.2 ml/10 ml double strength medium) and distributed aseptically in accurately measured quantities in $8 \times \frac{1}{2}$ in test tubes which had been cleaned in acid bichromate and sterilized with gauze covered plugs.

In some experiments this basal medium was replaced by one containing 33 mM phosphate buffer (prepared from

KH_2PO_4 (A R) with CO_2 free N NaOH added to give pH 7.3)

Procedure. Before inoculation, the tubes of medium were warmed in a well stirred water bath, thermostatically controlled at $35.0 \pm 0.1^\circ$. No attempt was made either to aerate or to secure anaerobic conditions. To avoid errors due to the continuation of germination during the sampling of a long series of tubes, experiments were set up by inoculating at timed intervals, usually 1 min. Sampling, by making tenfold dilutions in distilled water, also took place at corresponding intervals. Where initial counts were required, samples were taken just after inoculation, and the tubes of inoculated medium were returned to the water bath immediately after sampling. For $S + V$ counts, the tubes of diluent were cooled in ice water before sampling and were kept in the cooling bath after sampling until the contents were plated. For S counts, the tubes of diluent were heated to 60° before sampling and heating at this temperature was continued for 15 min. after sampling to destroy all germinated forms. The dilutions were then cooled and kept in ice water till the time of plating.

Except where stated otherwise, the inoculum was about 12,000 spores/ml. and the period of incubation was 30 min.

Viable counts and their accuracy. The principle of the surface plate technique of Miles & Misra (1938) was used with minor modifications in technical details. The composition of the medium for plating was 2% Evans peptone, 0.5% NaCl and 1.5% agar, pH 7.6. To ensure uniformity of successive batches of medium, it was made from a single batch of peptone, of which a sample had been shown to give satisfactory counts of the test organism, never significantly less, and, with some suspensions, many times greater than those on tryptic ox agar. Plates were poured without cooling the melted agar and were dried, inverted over the lids, for 90 min. at 60° . Dropping pipettes, delivering 42 drops/ml., were used to distribute 7 drops of appropriate dilutions over each of 3 plates. Plates were incubated either for 12 hr. at

37° or for 15 hr. at 30° , when the colonies were discrete and of uniform size, about 1 mm. in diameter, provided that the number on each plate did not exceed 250. In designing the experiments, control plates were inoculated so as to yield about 200 colonies from the spores which had failed to germinate after incubation in the deficient basal medium. With the usual inoculum, this was attained by plating, as described, 0.5 ml. of the tenfold dilution made in sampling. Samples showing a fall in count due to germination were plated at the same dilution.

Ideally, the chance of observing a particular value in a series of replicates should conform to a Poisson distribution, for which both the mean and the variance are estimated by the mean of the observations (Fisher, Thornton & Mackenzie, 1922). For a total control count of 500–700 colonies on the three plates, the standard error was thus about 25 colonies and the limits were of the order of $\pm 8\%$ with a probability, $P=0.95$. Ultimately, there were available for testing conformation to a Poisson series, 47 sets of quadruplicate controls and 21 sets of quintuplicate controls. With such a small series, it was necessary to group the data at both tails of the distribution, each amounting to 10% of the whole, to avoid expectations less than 5 in any group (Table 1). Owing to the presence of four aberrant values exceeding the theoretical 1% level, the χ^2 for deviations from the theoretical distribution could not therefore accurately summarize the whole of the available information. It indicated a larger probability, 0.5, for the occurrence of greater deviations by chance than 0.18 shown by the total χ^2 for variations within sets of replicates, the latter measure being very sensitive to the presence of a few excessively variable replicates, while giving little information on the general form of the distribution. Taking both criteria into consideration, it was considered justifiable to regard this sample of counts as conforming to a Poisson series, but in using the square root of a count as an estimate of its standard deviation, in order to assess the significance of differences, the precaution was

Table 1. Conformation of control counts to Poisson series

Probability of observing greater deviations by chance	No. of sets of replicates observed			No. of sets expected (N)	Difference ($n - N$)	$\frac{(n - N)^2}{N}$
	Sets of 4	Sets of 5	Total n			
1.00	0	0	0			
0.99	1	0	1			
0.98	2	2	4			
0.95	1	3	4	6.8	+2.2	0.71
0.90	5	2	7	6.8	+0.2	0.01
0.80	6	2	8	6.8	+1.2	0.21
0.70	6	5	11	13.6	-2.6	0.50
0.50	6	2	8	13.6	-5.6	2.30
0.30	8	3	11	6.8	+4.2	2.58
0.20	6	1	7	6.8	+0.2	0.01
0.10	1	0	1			
0.05	1	0	1			
0.02	1	0	1	6.8	+0.2	0.01
0.01	1	0	1			
0.00	3	1	4			
Total	47	21	68	68.0	0.0	6.33
Total χ^2 for deviations of counts from mean of set	175.4	69.3	244.7	—	—	—
Degrees of freedom (ϕ)	141	84	225	—	—	7
$\sqrt{2\chi^2 - \phi}$	1.07	1.15	0.93	—	—	—
Probability of observing greater deviations by chance	0.025	0.88	0.18	—	—	0.5

adopted of examining the data in a particular experiment for evidence of excessive variation

Comparison of potencies of active preparations The effects of preparations promoting the germination of spores in the presence of the basal medium were measured by the decrease in *S* count caused by the preparation in excess of the small decrease observed in the basal medium alone under the same conditions

The control *S* count, after incubation in the basal medium, was used as an estimate of the number of spores which had been subject to the influence of the active preparations

For comparison of potencies it was found that a convenient linear concentration relationship could be obtained by the probit-log concentration transformation (see Finney, 1947). Statistical justification of this procedure will be published elsewhere. Simple graphical methods sufficed, however, for general use

RESULTS

Preliminary experiments

The rate of germination of a small inoculum, about 1000 spores/ml, in the usual laboratory media, was so rapid that a high proportion of spores lost their resistance to heat during the time required to make the inoculation and take a sample. On the other hand, the rate was so slow in the synthetic medium of Gladstone (1939), that it was not measurable by the technique adopted. The proportion of spores which became sensitive to heat in a complex medium during a convenient experimental period could be controlled, however, by increasing the size of the inoculum, or by diluting the medium. The latter method was chosen in the first place, in order to keep the inoculum size as small as possible, and so avoid transferring to fresh medium unknown essential metabolites present in the seed. It is recognized that this could be no more than a temporary expedient, since investigation of the effect of a particular nutrient might be of little value if concurrent deficiencies of other nutrients occurred.

In order to gain some idea of the types of material essential for germination, the CCY medium of Gladstone & Fildes (1940) was used since its composition, though complex, can be subjected to some degree of controlled variation. The bulk of the amino-acids were supplied by an acid hydrolysate of casein, while those destroyed by acid hydrolysis, especially tryptophan, were supplied by a tryptic digest of the same protein. Other growth factors were supplied by yeast extract. Using this medium at 10% of the usual concentration (Table 2), it was shown that the tryptic digest was not essential, and that the small activity of the acid hydrolysate (Exp 1), with or without tryptic digest, was much enhanced by yeast, although the latter, in the presence of buffer, was inactive (Exp 2). The yeast extract was not replaceable by a mixture of known growth factors. Its fractionation was therefore undertaken with the object of identifying the active components.

Table 2 *The effect of constituents of CCY medium on germination*

(Basal medium, 0.05M glycerophosphate, pH 7.3. The additions were prepared as described by Gladstone & Fildes (1940) and the final concentrations in the medium were acid hydrolysed casein (AC), 0.07%, tryptic digest of casein (TC), 0.03%, yeast extract (Y) equivalent to 0.2% fresh yeast.)

Exp no	Additions to basal medium	Spore count at			
		0 min	15 min	30 min	60 min
1	None	446	—	—	362
	AC	447	462	376	198
	AC+TC	509	478	382	208
	AC+Y	340	140	58	30
	AC+TC+Y	483	131	74	29
2	None	608	—	520	—
	Y	571	—	502	—
	AC	646	—	372	—
	AC+Y	615	—	48	—

The diluted casein hydrolysate did not, however, provide a suitable basal medium for the assay of yeast fractions, since the degree of germination, even in the absence of yeast extract, was too high except at dilutions which may well have been suboptimal in several essential metabolites. The synthetic medium of Gladstone (1939) was also unsuitable, since, as in buffer, the activity of yeast was not observed in this medium. It became necessary, therefore, to devise a suitable basal medium.

Development of a basal medium for assay of yeast fractions

A suitable basal medium should, in general (1) contain optimal amounts of all essential nutrients except that to be assayed, and (2) usually be as free from the material to be assayed as possible. Condition (1) is satisfied when the biological effect in the presence of an active supplement is independent of the concentration of the basal medium within suitable limits. Condition (2) is satisfied when the effect in the basal medium alone is small.

After a number of trials, a hydrochloric acid hydrolysate of gelatin, supplemented by tyrosine and buffered by 33 mM-phosphate, pH 7.3, was found to allow a satisfactory response to yeast extract. In the presence of 0.28 mM L tyrosine, the optimum concentration of gelatin hydrolysate was 0.25% (Fig. 2). Lower concentrations of gelatin were little less effective, but higher concentrations were inhibitory. No advantage in this respect was offered by a hydrolysate of lower salt content made with sulphuric acid and treated with barium hydroxide to remove sulphate ions. A fivefold increase in the tyrosine concentration, supplied either as the pure isomer or as twice the amount of synthetic DL tyrosine, did not increase the extent of germination (Table 3). The initial counts showed that the presence

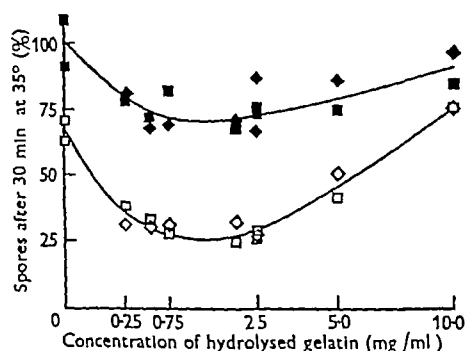


Fig 2 Optimum concentration of hydrolysed gelatin for germination in presence of 0.005% tyrosine A, basal medium with gelatin hydrolysed by 23% HCl, 0.75 hr at 120°, B, basal medium with gelatin hydrolysed by 10% H₂SO₄, 3 hr at 130°, A + yeast extract equivalent to 0.2% fresh yeast, B + yeast extract equivalent to 0.2% fresh yeast, To increase the range covered, the horizontal scale is proportional to the square root of the concentration

of tyrosine in the dilutions during heating did not significantly affect the capacity of the spores to develop colonies on plating For routine use, a

Table 3 Effect of tyrosine on germination in presence of acid hydrolysed gelatin and yeast extract

(The basal medium contained HCl hydrolysed gelatin, equivalent to 0.25% (w/v) gelatin, and yeast extract equivalent to 0.02% (w/v) fresh yeast in 33 mM phosphate buffer, pH 7.3)

Addition to basal medium	Concn of each enantiomorph (mM)	Spore count at	
		0 min	30 min
None	—	375	283
L-Tyrosine	0.28	381	106
	1.4	369	89
DL Tyrosine	0.28	384	99
	1.4	405	103

crude sample of L tyrosine, obtained as a by product from the preparation of tryptic digest of casein, was found to be as suitable as the pure preparations

Table 4 Effects of various metabolites on germination in acid hydrolysed gelatin and tyrosine, with and without yeast extract

(Basal medium, HCl hydrolysed gelatin equivalent to 0.25% gelatin, 0.28 mM L-tyrosine (crude) in 33 mM phosphate buffer pH 7.3)

Additions to basal medium	Concn (M)		Concn (M)
Cations		Tryptophan (L-)	0.5×10^{-6}
Ca ⁺⁺	1.0×10^{-3}		
Fe ⁺⁺	0.8×10^{-4}	Vitamins	
Mg ⁺⁺	0.5×10^{-3}	p Aminobenzoate	0.8×10^{-6}
Glucose	1.3×10^{-2}	Aneurin	2.0×10^{-6}
Guanine and pyrimidines		Ascorbic acid	0.4×10^{-3}
Cytosine	4.5×10^{-5}	Choline	0.4×10^{-6}
Guanine	3.3×10^{-5}	Inositol	0.4×10^{-3}
Thymine	4.0×10^{-5}	Nicotinic acid	0.7×10^{-6}
Uracil	4.5×10^{-5}	Pantothenate	2.0×10^{-6}
Sulphur compounds		Pimelate	0.7×10^{-4}
Cystine (L-)	1.0×10^{-4}	Pyridoxine	0.8×10^{-6}
Glutathione	1.0×10^{-3}	Riboflavin	2.0×10^{-6}

(Since the techniques gave control counts conforming to the Poisson distribution (Table 1), the standard error (s) of the difference between two counts (D) could be taken as the square root of the sum of the two counts Ratios D/s exceeding 1.96 were considered to show significant differences ($P=0.95$) and are given in heavy type)

Additions to basal medium	Initial spore count	D/s	After 30 min at 35°			
			No yeast		0.03% yeast	
			Spore count	D/s	Spore count	D/s
None	644	—	641	—	253	—
Cations	651	0.19	529	3.28	243	0.45
Glucose	638	0.17	593	1.37	296	1.84
Guanine and pyrimidines	654	1.10	648	0.19	348	3.88
Sulphur compounds	684	1.10	627	0.39	234	0.86
Tryptophan	642	0.06	584	1.63	247	0.27
Vitamins	665	0.59	605	1.02	286	1.42
All	679	0.96	638	0.03	303	2.13

In order to check the adequacy of the supply of essential factors in this basal medium, the effects of various known growth factors were tested, not only on the blank, but also on the germination with suboptimal amounts of yeast extract (Table 4) The materials tested included cations, glucose, guanine, pyrimidines, sources of organic sulphur, tryptophan and water soluble vitamins No pronounced effects were observed, but those which were statistically significant were the small inhibition of 15 % by guanine plus pyrimidines in the presence of yeast, and the small promotion of germination of 14 % by cations in the absence of yeast These effects could have no influence on the assay of yeast fractions, since the concentrations, at levels similar to those found optimal for growth by Brewer *et al* (1946), were at least 50 fold greater than those likely to be present in yeast extracts of much greater activity The cations, however, together with aneurin and glucose, were included in the basal medium, since they were essential for good growth and therefore, presumably, for survival of germinated forms Details for preparation of the medium are given in the section on Methods, p 354

Replacement of yeast factor by adenine derivatives

After the development of a suitable basal medium, the problem was pursued along two independent lines Boiled yeast extracts, or boiled autolysates up to twenty times as active as such simple extracts,

were fractionated to give preparations active at 2.5–5.0 $\mu\text{g/ml}$ These experiments are not described in detail, since the testing of materials known to be present in yeast extract showed activity in preparations of many nucleic acid derivatives (Table 5) A commercial sample of yeast adenylic acid was highly active, and a guanylic acid preparation also showed some activity, but adenine and guanine were inactive Preparations of yeast and thymus nucleic acids also showed some activity In a second experiment (Table 5) the purines were tested with and without ribose to see if the latter made the synthesis of an active compound possible Significant effects were not observed Fig 3 shows that the maximum

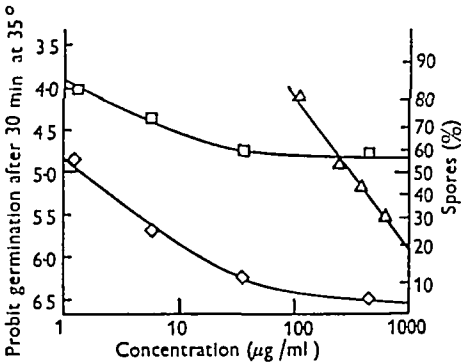


Fig 3 Effect of nucleotide preparations and yeast extract on germination Adenylic acid, \diamond , guanylic acid, \square , yeast extract, \triangle

effect was given by both adenylic acid and guanylic acid at a concentration of about 35 $\mu\text{g/ml}$ or 10^{-4}M , but the latter showed only 40 % germination, compared with over 90 % for adenylic acid The effect of adenylic acid, however, was qualitatively different from that of yeast, since twofold increase of yeast concentration increased germination from 45 to 70 %, whereas fivefold increase in adenylic acid concentration was necessary to produce a similar change in germination It must be emphasized that the preparations used up to this stage had been commercial products which had not been specially purified The activity may therefore have been due to impurities This was also suggested by the properties of the material in yeast extract, in particular the failure to get a barium salt insoluble in 67 % ethanol Further, during the course of work on the amino acids essential for germination (Hills, 1949), it was found that there was a five to ten fold increase in the activity of the sample of adenylic acid used (Table 6) in 5 mM aqueous solution at its own pH (3.1) during a period of about 6 months at room temperature In view of the probability of hydrolysis under these conditions, a commercial sample of adenosine was tested at the same time and found to have practically the same

Table 5 Effect of nucleic acid derivatives on germination in basal medium

(Ribose, where added, was $4 \times 10^{-4}\text{M}$ Other additions were $2.5 \times 10^{-4}\text{M}$, except yeast adenylic acid, 10^{-5}M , guanylic acid, 10^{-4}M and yeast extract equivalent to 375 μg fresh yeast/ml (Exp 1) or 500 $\mu\text{g/ml}$ (Exp 2))

Exp no	Additions to basal medium	Spore count after 30 min at 35°	
1	None	495	
	None	491	
	None	463	
	Adenine	492	
	Adenosine triphosphate	550	
	Adenylic acid	216	
	Guanine	490	
	Guanylic acid	302	
	Thymus nucleic acid	434	
	Yeast nucleic acid	265	
2	Yeast extract	201	
		Without ribose	With ribose
	None	724	766
	None	938	938
	Adenine	908	944
	Guanine	757	948
	Hypoxanthine	764	944
	Xanthine	892	826
	Adenylic acid	126	67
	Yeast extract	122	102

activity as the old solution of adenylic acid, i.e. five to seven times the activity of freshly made solutions of preparations of yeast adenylic acid from two different commercial sources. Adenine cytosine dinucleotide had the same activity as adenylic acid on a molecular basis, but a guanosine preparation showed much smaller activity.

Table 6 Concentrations of preparations of nucleosides and nucleotides for 50% germination under standard conditions

(The conditions were 30 min at 35° in the basal medium described on p 354. The concentration for a final spore count 50% of that in the basal medium was estimated from points in the neighbourhood of 50% survivors on the assumption that the probit-log concentration regression line was parallel to that of the standard yeast preparation, which gave 50% survivors at 131–151 µg/ml in these experiments.)

Addition to basal medium	Concn for 50% germination (µM)
Adenosine	0.8
Yeast adenylic acid	
Sample A, fresh solution	4.0
Sample A, 6 month-old solution	0.5
Sample B, fresh solution	5.5
Adenine cytosine dinucleotide	6.1
Guanosine	80

In view of the importance of tyrosine in germination and the similarity in the stability of tyrosine codecarboxylase (Gale & Epps, 1944) to the germination factor from yeast, it seemed likely that this coenzyme might be a possible biologically active impurity in commercial preparations of nucleic acid derivatives. A sample, kindly supplied by Dr E. F. Gale, was, however, found to be inactive even at 1 mg/ml.

From these observations it appears that the activities of nucleic acid derivatives might reasonably be accounted for by their content of adenosine, a preparation of which was the most active material tested.

Purification of adenosine

In order to complete the identification of adenosine as the active material, the changes in activity were examined (Table 7), following purification by recrystallizations from water without change in m.p. 224–226° (uncorr.). The final product, dried *in vacuo* over CaCl₂, gave the following analytical results (Weiler and Strauss) (Found, C, 44.4, H, 5.2, N, 25.6. Calc. for C₁₀H₁₃N₅O₄, C, 44.9, H, 4.9, N, 26.2%). Levene & Bass (1931) gave m.p. 229°, with 1.5 mol water but the conditions of drying were not stated. Fractions D and E, the mother liquor and crystals, respectively, from the third recrystallization, had closely similar activities at all four levels tested. The original commercial specimen A and the mother liquors B and C, from the first and second recrystallizations, respectively, were less active, except for the two lowest levels of preparation C in Exp. 1. The χ^2 test was therefore applied on the assumption that the best estimate for each of the levels tested was the mean of the corresponding values for D and E. Highly significant differences ($P < 0.001$) were observed for A, B and C in both experiments, but the deviations for D and E together were not significant ($P = 0.21$).

Sterilization of adenosine

In the experiments reported so far, the adenosine had been sterilized by autoclaving in aqueous solution (15 lb./in², 20 min.). The purest preparation E

Table 7 Effect of recrystallization on activity of commercial sample of adenosine in promoting germination

(The significance of differences was tested by χ^2 for the deviations of all points for each preparation from the means of corresponding points for preparations D and E. The control counts, without addition of any preparation, were 668.4 and 597.8 in Exps 1 and 2 respectively, each the mean of quintuplicate.)

		Spore count after 30 min. in basal medium + preparation				
		A	B	C	D	E
		Mother liquors from			Solid phase	
		Original sample	1st recrystallization	2nd recrystallization	3rd recrystallization	from 3rd recrystallization
Exp no	Adenosine concn (µg/ml)					
1	0.188	537	558	515	523	515
	0.470	425	426	334	370	330
	1.175	318	278	301	267	239
	2.94	163	191	193	151	160
2	0.188	528	608	637	393	397
	0.470	362	386	401	304	331
	1.175	243	290	175	132	164
	2.94	156	157	148	114	130
χ^2		153.8	303.7	296.6	10.76	
Probability of difference due to chance		0.001	0.001	0.001	0.21	

was tested after sterilization both by autoclaving and by Seitz filtration (Table 8). Exp. 1 showed no significant differences at any concentration, and although Exp. 2 suggested that the filtered solution was less active at each concentration tested, only at the highest concentration did the difference appear to be significant by a simple χ^2 test. Examination of replicate counts in this experiment showed greater

Effect of adenosine on vegetative and spore counts

Except for a few early experiments in complex media it has been assumed throughout this work that the fall in spore count on incubation in culture media was due to germination. Omission of controls on possible changes in the total count was justified in

Table 8 *Effect of purified adenosine on germination after sterilization by autoclaving or Seitz filtration*

(Thrice recrystallized adenosine was used (Preparation E, p. 359). Sterilization was carried out in 2 mM solution, autoclaving for 20 min. at 15 lb./in.², and Seitz filtration through a Ford Sterimat S.B., 10 ml. through a pad 3.6 cm. in diameter. Control spore counts with no adenosine in the basal medium were 392.6 and 922.2 for Exps. 1 and 2 respectively (sum of three plates, each sum the mean of quintuplicate determinations). The period of incubation was 30 min. at 35°.)

Spore counts with adenosine									
Exp no	Concn of adenosine (μ M)	Autoclaved		Filtered		χ^2 $\frac{(n_1 - n_2)^2}{n_1 + n_2}$	Degrees of freedom (ϕ)	Probability of greater difference due to chance (P)	
		Single plates	Sum (n_1)	Single plates	Sum (n_2)				
1	0.6	81	289	82	264	1.13	1	—	
		99		89					
		109		93					
	1.8	49	159	56	179	1.18	1	—	
		52		61					
		58		62					
	6.0	40	123	37	117	0.15	1	—	
		41		39					
		42		41					
2	0.6	219	692	235	745	1.95	1	—	
		231		241					
		242		269					
	1.8	133	450	150	490	1.70	1	—	
		148		152					
		169		188					
	6.0	60	211	65	258	4.71	1	0.03	
		74		89					
		77		104					
				10.82					6
			1024		2053		4.18	1	0.04
	Analysis of variance								
				ϕ	χ^2	F	P		
	Treatments		1	4.18	3.20	0.09			
	Experiments		1	3.77	2.89	>0.1			
	Concentrations		2	2.35	0.90	>0.1			
	Interaction		2	0.52	0.20	>0.1			
				10.82					
	Plates		24	31.38					

variability than usual ($P=0.14$), especially for those counts showing the apparently significant difference between autoclaved and filtered solutions. Partition of χ^2 in the form of an analysis of variance and application of a variance ratio test showed that the difference was not, in fact, significant ($P=0.09$). The conclusion is that if any loss of activity occurred on autoclaving it must be equal to the loss by adsorption on Seitz filtration. There is no evidence that the activity of adenosine was due to breakdown on autoclaving.

the assay of preparations, since the observation of such controls would have doubled the experimental work, complicated the computations of activity and the errors of random sampling would have reduced the value of any adjustments which such changes might have made in the probit. Table 9 shows that the effect of adenosine was solely on the loss of heat resistance of spores, the 30% fall of the $V+S$ count on incubation was independent of the addition of adenosine to the basal medium, and whilst the proportion of heat resistant organisms remained

practically 100% after incubation in the basal medium, it fell to 11% in the basal medium plus 2 μ M adenosine. The remaining 89%, which were no longer spores since they had become heat sensitive, must be regarded as having germinated since they were still viable, giving visible colonies on plates of a suitable nutrient medium. Adenosine alone was ineffective in the absence of the basal medium.

Table 9 *Effect of adenosine on vegetative and spore counts*

(Sample equivalent to 0.05 ml undiluted culture)

Medium	Count of sample			
	Before incubation		After 30 min at 35°	
	Vegetative + spore	Spore	Vegetative + spore	Spore
Basal	688	680	498	492
Buffer + 2 μ M adenosine	702	694	560	467
Basal + 2 μ M adenosine	719	694	472	52

DISCUSSION

The importance of nucleic acid derivatives in bacterial nutrition was first shown by Richardson (1936), when he reported the need of *Staphylococcus aureus* for uracil during anaerobic growth, although the organism was capable of synthesizing this essential pyrimidine aerobically. Subsequent workers have established the need for nucleic acid derivatives by other organisms. In some cases, complex derivatives have been more effective than simple purines and pyrimidines, e.g. with *Neurospora* mutants (Loring & Pierce, 1944) or *Lactobacillus gayonni* (*L. fermenti*) (Hutchings, Sloane & Boggiano, 1946).

The mode of action of adenosine in germination, however, is at present obscure, since nothing is known either of the biochemical or biophysical changes which occur in the spore during germination, or of the metabolism of adenosine by the spore. Adenosine can be hydrolysed and deaminated by various non-spore-forming organisms (Lutwak Mann, 1936) and can stimulate the deamination of adenine by *Escherichia coli* (Stephenson & Trim, 1938) or of serine by *Clostridium welchii* (Woods & Trim, 1942). It would be of interest to examine the extent to which spores may catalyse such reactions as well as those of the type, purine riboside + phosphate \rightleftharpoons purine + ribose 1-phosphate, described in animal tissues (Kalckar, 1945). Such processes involving ammonia and phosphate transfer might provide a link between the stimulation of anthrax spores by adenosine and that of *Phycomyces* spores by guanine or hypoxanthine (Robbins & Kavanagh, 1942a, b).

A distinction must be drawn between the requirements for germination and those for growth. The

synthetic ability of cells emerging from a dormant state may be less than those of an actively growing culture (cf. Brown, 1946). The requirements for germination would thus tend to be more exacting than those for growth, as shown by the stimulation of germination but not of growth, by adenosine in the case of *B. anthracis*. In germination, with changes in a single cell without multiplication, it may be more difficult to show a requirement for a particular nutrient, since the cell may carry a supply, though insufficient for optimal activity, and the amount available for each cell cannot be markedly reduced as during growth. This may be the case with pyridoxal phosphate, an important coenzyme in metabolism of amino acids, including that of tyrosine (see Gale, 1946), however, although tyrosine was important in the germination of *B. anthracis* even high concentrations of the coenzyme had no influence. In growth, involving perhaps a million-fold increase in cell substance, provided that the cell is sufficiently equipped with essential metabolites for initiation of the process, the increased synthetic ability of the actively metabolizing culture may be capable of supplying essential materials which could not be synthesized by the germinating spore. In this way it is possible to explain the growth of *B. anthracis* in synthetic media not containing adenosine (Gladstone, 1939, Brewer *et al.* 1946). In such media growth is due to the germination of only a small proportion of the inoculum in the early stages, presumably under the influence of traces of adenosine in the cells. This proportion is frequently not detectable by the comparatively crude viable count technique. Increased germination cannot be excluded after changes have been produced in the medium by the actively growing culture, but it is masked by formation of fresh spores.

Although adenosine was the most active material tested in a high degree of purity, commercial preparations of yeast nucleic acid and its derivatives showed some activity. These materials were not purified to show how far the activity was specific for adenosine and how far it was due to related compounds. Adenine, however, was inactive, so that the specificity was greater than that of the *Neurospora* mutant described by Pierce & Loring (1945), for which the maximum growth rate was largely dependent on the amount of adenylic radical available rather than its state of combination. In view of the high sensitivity of the germination of *B. anthracis* to adenosine, this process might be made the basis of a microbiological assay. Less than 0.5 μ g could be detected and a three-point assay could be carried out on as little as 2 μ g without modifying the experimental technique already used. Since only 1% of the actual sample available was used in plating, the method could be made even more sensitive by the use of micropipettes. Of established

methods at present available, the guinea pig auricle method of Drury, Lutwak-Mann & Solandt (1937) requires 1–2 μg , while the use of a specific deaminase required about 1 mg unless the reaction is followed by ultraviolet spectrometry (Kalckar, 1947) when the amount may be reduced to 0.1 μg . The results given in Table 6 suggest an adenosine content of about 150 mg/100 g fresh yeast for the best yeast extract used in this work. Ostern, Terszakowec & Hubl (1938), using the deaminase method, found 360 mg/100 g dialysed acetone dried yeast after 20 hr autolysis, and this was equivalent to 60% of the adenine of the original cell nucleic acid. The amount found by the effect on germination therefore does not exceed the maximum expected from the work of Ostern *et al.* (1938), but a closer comparison is not possible on account of the effects of autolysis and other conditions.

SUMMARY

1 The metabolites essential for the rapid germination of spores of *Bacillus anthracis* have been

studied, using loss of resistance to heat as a measure of germination, determined by viable count.

2 A suitable deficient medium for the assay of promoters of germination contained 0.25% acid hydrolysed gelatin and 0.005% L-tyrosine. Higher concentrations of hydrolysed gelatin were inhibitory.

3 The slow germination in the basal medium was accelerated significantly by yeast extract equivalent to 0.01% fresh yeast.

4 The yeast extract was replaceable by a purified preparation of adenosine which gave a significant effect at a concentration of 0.2 μg /ml.

I have great pleasure in thanking Dr D. W. Henderson and Dr D. D. Woods for careful criticism of this paper. The experimental work was carried out, with the technical assistance of Cpl W. Bailey, R.A.M.C., while the author was a member of the Scientific Staff, Medical Research Council. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

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Chemical Factors in the Germination of Spore-bearing Aerobes. The Effects of Amino-acids on the Germination of *Bacillus anthracis*, with some Observations on the Relation of Optical Form to Biological Activity

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(Received 29 March 1949)

The first paper of this series (Hills, 1949) has shown that, in a basal medium in which essential amino-acids are supplied by a gelatin hydrolysate and tyrosine, the rate of germination of spores of *Bacillus anthracis* was greatly increased by boiled yeast extract and that the extract could be replaced by adenosine at a concentration of 10^{-4} M or about 0.25 mg/l. It thus became possible to study the effects of amino acids on germination, since the optimum concentration expected for many of them, of the order of 100 mg/l. on the basis of the optimum concentration of gelatin hydrolysate (0.25%), was much greater than that of the adenosine. Observations on amino acid requirements were therefore not likely to be vitiated by trace impurities in the adenosine, which was, necessarily, a natural product.

Difficulty was encountered in the study of amino-acid requirements, however, since the synthetic medium for the growth of *B. anthracis* used by Gladstone (1939), and since modified by Brewer, McCullough, Mills, Roessler, Herbst & Howe (1946) supported a rate of germination, even in the presence of yeast extract or adenosine, too small for detection by viable counts, though some germination must have occurred since the strain used grew satisfactorily on the modification of the latter authors. Gladstone (1939) had shown, however, that growth in a modification of his medium containing fourteen amino-acids was inhibited by leucine, isoleucine and valine separately, but was stimulated when these three amino acids were present in appropriate proportions. In a further simplified medium, containing a total of eleven amino acids, these three were essential. It seemed possible, therefore, that the feeble germination in synthetic media, and the inhibition of germination which occurred with concentrations of gelatin hydrolysate above the optimal (Hills, 1949), might both be due to an unsuitable balance of amino acids. Work was therefore begun by attempting to simulate the effect of excess gelatin hydrolysate by means of amino acids, with the hope that this would reveal the cause of failure to germinate in the amino acid medium.

METHODS

Technique Experimental methods, based on the determination of viable counts by the surface plate technique of Miles & Misra (1938), were as described previously (Hills, 1949). It is again emphasized that all counts were viable counts and the abbreviation ($S + V$ count) is used for the sake of brevity to denote a count determined so as to include both spore and vegetative cells. The period of incubation was 30 min. at 35°, except where otherwise stated.

Materials These were largely of commercial origin, but the adenosine and inorganic constituents of the media were purified by recrystallization thrice from water. One specimen of D-alanine was kindly presented by Roche Products Ltd. A second specimen, for which the author thanks Dr H. N. Rydon, was prepared by resolution of the benzoyl derivative of commercial synthetic DL-alanine with brucine, and had $[\alpha]_D^{25} = -7.8^\circ$ at c, 1.342 g. hydrochloride/100 ml. N HCl, indicating 90% optical purity. A specimen of L-alanine, also prepared by Dr Rydon by resolution of the benzoyl derivative with strychnine, was optically pure, having $[\alpha]_D^{25} = +10.7^\circ$ at c, 1.684 g. hydrochloride/100 ml. N HCl.

The author thanks Dr Rydon also for a specimen of synthetic DL-tyrosine.

Pyruvic acid was purified by vacuum distillation and stored in the cold as 5M solution (Wendel, 1932). Lactate was purified by recrystallization of the Zn salt and Zn was removed by Na_2CO_3 .

Concentrations of materials have been expressed as the molarity of a single enantiomorph, the total concentration of a DL-mixture was thus twice that given.

Statistical interpretation of results Since the technique was shown in the previous paper to give counts conforming to Poisson distributions, it was possible to take each count as an estimate of its sampling variance, and the sampling variance of the difference between two counts at the same dilution was therefore equal to the sum of the counts. This simple technique sufficed for the assessment of the differences between counts in the same experiment by well known methods as described, for example, by Fisher (1946) or Davies (1947).

In comparing the proportions germinating in separate experiments it is necessary to calculate the variance of the proportion. This is not given by the usual formula based on the binomial distribution, since the observations are not the numbers germinating and failing to germinate, but are estimates n of the spores subjected to a particular treatment.

and s of those failing to germinate, both determined on equal portions. Since these vary independently according to Poisson distributions, the sampling variance of $q (=s/n)$, the proportion of spores not germinating, is given by

$$V(q) = (\partial q / \partial s)^2 V(s) + (\partial q / \partial n)^2 V(n)$$

If n is the mean of l replicates and s that of l replicates

$$V(n) = n/l, \quad V(s) = s/l$$

and $V(q) = (s/l + s^2/l^2)/n^2$ or $(q/l + q^2/l)/n$

The second term of these expressions for $V(q)$ is usually small, contributing less than 10% to the standard error if $l=5l$. This frequently arises, since the initial counts for a number of independent treatments rarely differ significantly, and may therefore be pooled to give the best estimate of n .

In studying the effects of amino acids as inhibitors of germination (Table 8), uncontrollable variations in conditions led to variations in the degree of germination of the uninhibited control in the different experiments. It was then found advantageous to normalize the data by transformation of proportions to probits (see Finney, 1947). Since the probit x is related to the proportion q so that $dq/dx = z$, the ordinate of the normal curve corresponding to the proportion q , the sampling variance of x is given by

$$V(x) = (dx/dq)^2 V(q) = (q/lz^2 + q^2/lz^2)/n$$

The sampling variance of the difference between two probits, based on the same estimate of n , is given by

$$\begin{aligned} V(x_1 - x_2) &= V(x_1) + V(x_2) - 2 \text{Cov } x_1 x_2 \\ &= V(q_1)/z_1^2 + V(q_2)/z_2^2 - 2 \text{Cov } q_1 q_2 / z_1 z_2 \\ &= \{q_1/l_1 z_1^2 + q_2/l_2 z_2^2 + (q_1/z_1 - q_2/z_2)^2/l\}/n \end{aligned}$$

If the reciprocal of this theoretical sampling variance was used to weight the probit differences derived from the data of Table 8, an χ^2 test showed that the variations between experiments were greater than expectation, but χ^2/ϕ , where ϕ is the number of degrees of freedom involved in determining the weighted mean of corresponding probit differences, showed no significant heterogeneity (Bartlett's test) due to the particular experiment involved ($P=0.7$), the amino acid tested for inhibition ($P=0.9$), or its concentration ($P=0.7$). It was thus possible to use a pooled estimate of the variance, χ^2/ϕ , based on the data as a whole, and the larger number of degrees of freedom available from this estimate allowed greater precision in the application of the t test to the significance of the weighted means.

This advantage of uniformity of variance over a wide range justified the use of the probit transformation in spite of the disadvantage that, on approaching complete inhibition of germination, the probit tends to infinity with zero weight. The significance thus becomes indeterminate unless an estimate from the regression of probit on a function of concentration of inhibitor is available. This was not necessary for the present purpose since, in practice, the significance of such large inhibitions was not in doubt. By taking the minimum expected probit X as that observed at a lower concentration of inhibitor, it was possible to calculate a minimum value for the working probit, $x = X + (q - Q)/Z$ (as defined by Finney, 1947), where Q, Z are the proportions and ordinate of the normal curve corresponding to probit X , and q is the observed proportion not germinating at the higher concentration. The weight to be used with this working probit is that corresponding to Q and not to q . It will be observed that this technique allows appropriate weight to be given even to observations where errors of

random sampling lead to an estimate of q greater than 1 (e.g. with D alanine at 20 μM , Table 8, Exp 3). It has been applied to this and three other observations in Table 8, since their omission leads to a lower estimate for variance for the data as a whole.

RESULTS

Replacement of hydrolysed gelatin in basal medium by a synthetic mixture of amino-acids. Preliminary experiments on the toxicity of amino acids in the gelatin-tyrosine yeast medium, showed that leucine, isoleucine and valine, which Gladstone (1939) had shown to be inhibitory to growth under certain conditions, did not inhibit germination under the conditions used, the concentrations being those which Gladstone had found to be effective. Of the eleven amino acids present in the simplest form of his medium, however, D,L alanine was found to be strongly inhibitory, the activity being due to the D component. The inhibition due to excess gelatin hydrolysate was probably not due to this cause, since that due to $10^{-4} M$ D alanine was reduced from 95 to 25% by increasing the hydrolysed gelatin concentration from 0.05 to 0.30%. It is probable that the inhibition due to high concentrations of hydrolysate was largely due to glycine, at 10 mM it reduced germination in 0.25% hydrolysate from 70 to 35% compared with a reduction to 50% when the concentration of hydrolysate was doubled. Taking 24% as the glycine content of gelatin (Block & Bolling, 1945), this corresponded to an increase of 8 mM in glycine concentration.

Since it now seemed likely that failure of a large proportion of spores to germinate in a synthetic amino acid medium was due to D,L alanine, germination was tested in a medium containing eighteen amino acids, including threonine, at the concentrations used by Gladstone (1939), but with D,L alanine replaced by the L form. Other constituents of the medium were modified according to Brewer *et al.* (1946). Table 1 shows that this medium allowed a slow fall in spore count. In this medium the fall was much more rapid with the addition of a preparation of yeast adenylic acid, which was used at this stage before it was realized that its activity was probably due to its content of free adenosine. In buffer the fall was much slower and was not markedly stimulated by the adenylic acid. Although the combined count of vegetative cells and spores also fell slowly, the fall in spore count has been regarded as the best measure of germination, since at 0.5 hr when no significant fall in $S + V$ count had occurred, the spore count in the best medium had fallen by over 90%. The fall in combined count on prolongation of incubation showed that a significant proportion of the heat sensitive forms slowly became non-viable. This applied especially to those media which did not support growth (as shown by the count at 73 hr) so that in these media the proportion

of spores remained high. Nevertheless, it would be misleading to take the fall in the proportion of spores as an index of germination, since this proportion may increase, as in the 'eighteen amino acids' medium, with adenylic acid, due to a fall in the $S + V$ count while the number of spores actually remains practically constant.

medium of Exp 2, containing aspartate and glutamate in addition, but the three excluding glycine were as effective as that basal medium.

If the concentrations of either alanine or tyrosine were increased above 0.5 mM, the germination increased but about 1% spores did not germinate, even at 1–5 mM. At submaximal concentrations the

Table 1 *Germination and growth of Bacillus anthracis in a synthetic medium*

(The 'eighteen amino acids' medium was that of Gladstone (1939), including salts and glucose, with replacement of DL-alanine by L-alanine (5.6×10^{-4} M), and the addition of DL-threonine (3×10^{-5} M), aneurin (10^{-7} M), CaCl_2 (5×10^{-4} M), and NaHCO_3 (10^{-2} M). Yeast adenylic acid (y a a), where added, was 10^{-2} M.

The inoculated media, 10 ml in 6 in \times $\frac{1}{2}$ in tubes, were incubated for the first 5 hr in a water bath, at $35.0 \pm 0.1^\circ$, samples of 1.0 ml being removed at intervals for counting. Subsequently the remainder of the culture was aerated by sloping on a rocker in the hot room in such a way that the tubes passed in a vertical plane through 35 oscillations/min of amplitude about 5° , the minimum slope being as nearly horizontal as possible.

$S + V$ = viable count of spores and vegetative cells, S = spore count.)

Time (hr)	m/30 phosphate pH 7.3				'Eighteen amino acids'					
	No y a a		With y a a		No y a a		With y a a			
	(S + V)	(S)	(S + V)	(S)	(S + V)	(S)	(S + V)	(S)		
	Counts (× 10 ³)									
0.0	8.6	8.7	8.4	8.4	8.3	7.5	8.0	6.5		
0.5	7.5	7.6	8.0	6.2	7.3	5.9	7.7	0.5		
1.5	7.2	5.9	6.7	4.1	6.1	3.1	6.0	0.1		
3.0	5.9	5.4	6.1	4.6	5.9	2.5	3.8	0.1		
5.0	5.8	5.0	5.7	4.2	4.4	1.0	4.1	0.1		
	Counts (× 10 ³)				Counts (× 10 ⁷)					
72	—		3.4	—	2.0	—		7.5	—	4.7

Determination of essential amino-acids It was now comparatively simple to determine the amino acids essential for germination by omission of groups of them in turn as shown in Table 2. In Exps 1 and 2 the basal media were adequate, and the fall in spore count was not increased by any of the thirteen amino acids added. The inhibition by D-alanine was shown in Exp 1, and leucine, isoleucine and valine were together partially inhibitory in the basal medium of Exp 2. Exp 3 shows that the group containing glycine and L-alanine was effective with phenylalanine and tyrosine as the only amino acids in the basal medium, but aspartate and glutamate were ineffective. This latter mixture was also ineffective in the presence of glycine and L-alanine (data not tabulated). The inhibitory effect of glycine was more marked in these mixtures containing few amino acids, so that in this experiment where it was present in both effective mixtures little germination was observed in 0.5 hr and it was necessary to prolong the experiment for 2 hr before more than 50% germination was observed. Exp 4 shows that L-alanine and tyrosine together were effective, phenylalanine being stimulatory in their presence and glycine inhibitory. These effects were shown most clearly after 1 hr incubation. The four amino acids together were less effective than the basal

actual germination observed was somewhat variable, but a typical experiment (Table 3) showed that 100–150 μM was adequate for over 50% germination. In another experiment with 300 μM alanine, 300 μM tyrosine and 1 μM adenosine (now used in place of adenylic acid) the germination, 75%, was practically as great as the 90% observed in the gelatin tyrosine medium with the addition of the same concentration of adenosine. A tenfold dilution of the CCY medium of Gladstone & Fildes (1940) was rather more effective, only 1% of the spores remaining heat resistant as with higher concentrations of alanine and tyrosine. With tryptic digest of beef, on the other hand, even at a dilution tenfold greater than that usual in culture media, the germination of the small inocula used was so rapid that the spore count fell about 75% during the 1–2 min required for mixing the inoculum with medium at 35° and transferring a sample to the diluent at 60° . Since no significant fall occurred with the other media under these conditions, it must be concluded that the broth contains nutrients, so far unidentified, which can increase the rate of germination much beyond that attainable with those nutrients now known to be effective.

Inessential components of the medium The omission of aneurin, glucose or bivalent cations from the

Table 2 *Amino acid requirements for germination*
(All basal media contained salts and aneurin as in Table 1)

Exp no	Time (hr)	Basal medium		Additions		Spores not germinated	
		Constituents	Concn (μ M)	Substances	Concn (μ M)	(%)	(s D)
1	0.5	As in Table 1 with omission of cystine, methionine and tryptophan, with yeast adenylic acid	5	None		4.9	1.0
				L-Cystine	250	4.7	1.0
				DL-Methionine	200	10.9	1.5
				Two above		6.3	1.1
				Two above + DL-Serine + DL-Tryptophan	1000 1200	6.4	1.1
				Four above + D-alanine	280	88.9	3.1
				None		25.3	1.7
				DL-Leucine + DL-Isoleucine + DL-Valine	570 380 130	63.8	3.8
				L-Arginine + L-Histidine + DL-Lysine	500 250 270	37.0	2.9
				DL-Threonine + DL-Proline + DL-Hydroxyproline	30 440 380	41.8	3.0
2	0.5	Glycine	3300	None above		46.7	3.2
		L-Alanine	560	None		68.7	3.5
		DL-Aspartate	750	Glycine + L-Alanine	3300 560	34.0	2.5
		DL-Glutamate	300	DL-Aspartate + DL-Glutamate	750 300	73.8	3.7
		DL-Phenylalanine	300	Four above		32.2	2.4
		DL-Tyrosine	280	None		90.4	4.4
		Yeast adenylic acid	2	Glycine	3300	93.5	4.5
				L-Alanine	560	39.9	2.9
				DL-Phenylalanine	300	89.8	4.4
				Glycine + L-Alanine	3300 560	77.9	4.0
3	2.0	DL-Phenylalanine	300	Glycine + DL-Phenylalanine	3300 300	92.1	4.5
		DL-Tyrosine	280	L-Alanine + DL-Phenylalanine	560 300	10.7	1.5
		Yeast adenylic acid	2	Three above		55.3	3.4
				None		12.9	1.6
4	1.0	DL-Tyrosine	280				
		Yeast adenylic acid	2				
	1.0	As Exp 2					

Table 3 *Effect of concentrations of L-alanine and tyrosine on germination*

(The basal medium contained glucose, salts and aneurin as in Table 1, together with 1.5×10^{-6} M yeast adenylic acid)

DL Tyrosine (μ M)	0	10	30	100	300
L-Alanine (μ M)	Spore count (in 0.05 ml.) after 30 min at 35°				
0	588	550	600	552	429
	607	—	—	—	—
50	565	600	581	148	94
150	483	478	386	91	39
500	469	472	248	66	16

medium had no significant effect in an experiment in which the total number of viable organisms,

$98 \pm 3\%$ spores ($P = 0.95$) initially, fell by $32 \pm 4\%$ in media permitting a fall in spore count of $90 \pm 2\%$ independently of the presence or absence of these constituents. In the absence of any one of the remaining constituents, adenosine, L-alanine or tyrosine, germination was much reduced, the $S + V$ count falling to $75.5 \pm 4\%$ of the initial $S + V$ count and the spore count to $67 \pm 4\%$. This experiment confirms the cause in the fall in spore count as germination even in the simplest medium promoting such a fall. Replacement of the phosphate buffer by 25 mM sodium bicarbonate in equilibrium with 5% carbon dioxide in air, showed that phosphate was not essential but was probably stimulatory, giving 98% germination compared with 67% in medium

buffered by sodium bicarbonate, though the corresponding blank figures for the buffers alone were 12 and 31% respectively.

Replacement of tyrosine by analogue Table 4 shows a typical experiment on the effect of replacing tyrosine by phenylalanine or dihydroxyphenylalanine. Tyrosine was active at 150 μ M and the other two inactive at this level but active at 500 μ M. In all cases the D form present in the DL mixture appeared

Table 4 Effects of concentration and optical form of aromatic amino-acids on germination in the presence of adenosine and L-alanine

(The basal medium contained 33 mM phosphate, pH 7.3, 1.5 μ M adenosine and 500 μ M L-alanine. Dihydroxyphenylalanine was sterilized as 5 mM solution in 0.02N HCl by Seitz filtration just before use.)

Concn. of added amino-acid (μ M)	Spore count (in 0.05 ml) after 30 min at 35° with					
	Phenylalanine		Tyrosine		Dihydroxyphenylalanine	
	L	DL	L	DL	L	DL
500	286	313	178	183	337	280
150	490	425	332	255	555	441
50	536	517	539	438	552	459
0	494, 465, 430					

Table 5 Effects of analogues of tyrosine on germination in the presence of adenosine and L-alanine

(The basal medium contained 33 mM phosphate, pH 7.3, 6 μ M adenosine and 500 μ M L-alanine. Just before use the materials added were dissolved in 0.02N HCl, Seitz filtered and added to the basal medium containing the amount of NaOH required for neutralization.)

Addition to basal medium (500 μ M)	Viable counts (in 0.05 ml)			
	0 min		30 min	
	S-I	Spore	S-I	Spore
None	596	546	494	441
D-Hydroxytyrosine	596	501	460	341
Tyramine	579	525	480	295
Thyroxine	582	537	485	271
DL Dihydroxyphenylalanine	579	531	497	231
Adrenaline	593	504	502	192
DL Phenylalanine	603	527	490	151
DL Tyrosine	536	512	486	96

to have no marked effect on the activity. Table 5 shows the effects of some other analogues of tyrosine, all showing some activity. The case of adrenaline, with activity of the same order as phenylalanine, calls for special comment since it showed that an unsubstituted α -amino group was not essential for activity. Controls showed that these compounds influenced only the spore count after incubation, variations in the final S-I count and in the initial S-I and spore counts being within the errors of random sampling.

Specificity of alanine The stereochemical specificity of the enantiomorphs of alanine has already been indicated since it was necessary to replace DL-alanine by its L component before germination was observed in a synthetic medium. Table 6 shows that

Table 6 Specificity of L-alanine in promoting germination in the presence of adenosine and tyrosine

(The basal medium contained 33 mM phosphate, pH 7.3, 1.5 μ M adenosine and 500 μ M tyrosine. The concentrations of the additions to the basal medium were NH₄Cl and pyruvate, 5 mM; DL lactate and β -alanine, 2.5 mM; L-alanine, 0.5 mM.)

Additions to basal medium	Spore counts (in 0.05 ml)	
	0 min	30 min
None	664	547
NH ₄ Cl	647	589
Lactate	653	596
Lactate + NH ₄ Cl	637	572
Pyruvate	668	589
Pyruvate + NH ₄ Cl	633	577
β -Alanine	661	480
L-Alanine	626	62
L-Alanine + NH ₄ Cl	641	34
L-Alanine + lactate	659	35
L-Alanine + pyruvate	645	166

Table 7 Inhibition of germination by D-alanine

(The basal medium contained 33 mM phosphate, pH 7.3, 5 μ M adenosine and 500 μ M DL-tyrosine.)

Alanine (μ M)		Other additions	Spore count (in 0.05 ml)	
L	D		0 min	30 min
0	0	None	974	818
0	15	None	—	898
0	50	None	—	908
0	150	None	914	916
500	0	None	—	136
500	0	Brucine (20 μ g/ml)	845	248
500	0	Strychnine (20 μ g/ml)	897	231
500	15	None	—	403
500	50	None	—	655
500	150	None	—	836
1500	0	None	—	89
1500	15	None	—	142
1500	50	None	—	227
1500	150	None	—	461
5000	0	None	926	84
5000	15	None	—	73
5000	50	None	—	111
5000	150	None	910	385

L-alanine was not replaceable by β -alanine, nor by pyruvate or lactate, with or without ammonium chloride as a source of nitrogen. None of the last three was inhibitory in the presence of L-alanine, so that inhibition, as by D-alanine, did not occur with the corresponding form in DL-lactate. Reversal of D-alanine inhibition by L-alanine is shown in Table 7, a ratio of 10–30 molecules of promoter to one molecule of inhibitor giving about 50% germination.

Controls showed that traces of the alkaloids used in resolution could not be responsible for the inhibition, since in the presence of 500 μ M L alanine, 15 μ M-D alanine (1.35 μ g/ml) reduced germination by more than twice as much as 20 μ g/ml brucine or strychnine

Inhibition by other amino acids Indications of inhibition by glycine and by leucine, isoleucine and valine together had already been seen in determining

significantly inhibitory at 5 mM, but not at 1.5 mM, while with D glutamate and DL proline the inhibition at 5 mM was barely significant ($P \approx 0.05$). At this concentration D leucine, DL lysine, D phenylalanine and DL threonine showed no significant effect. Since D alanine was significantly inhibitory ($P = 0.01$) even at 5 μ M, the problem of the weaker inhibitions and their relation to stereochemical form has not been pursued further.

Table 8 *Inhibition of germination by amino acids*

(The basal medium contained 33 mM phosphate, pH 7.3, 2 μ M adenosine, 300 μ M L alanine and 300 μ M DL-tyrosine)

Exp. no.	1	2	3	4	5	6	7	
Initial spores (in 0.05 ml)	1	12	3	17	10	12	10	
No. replicates	Mean count	1017	650	816	516	570	493	402

Added to medium		Spore count after 30 min at 35°							Probit final spores		Probability of greater difference in one direction due to chance	Spores corresponding to probits (%)	
Amino acid	Concn. (μ M)								Weighted mean	S.E.		Mean	Difference
None	—	216	250	307	206	75	174	45	4.40	0.14	—	27	± 7
		204	237	287	221	95	154	33					
		198	—	—	208	—	—	—					
									Weighted mean difference				
D Alanine	1.5	—	542	—	330	—	156	—	+0.39	0.32	0.12	42	+14
	5	—	576	728	406	—	252	—	+0.85	0.35	0.010	60	+33
	20	—	—	869	416	—	—	—	>1.57	0.38	<0.001	83	+56
	60	944	—	—	—	—	—	—	+2.29	1.33	0.026	96	+69
	200	904	—	—	—	—	—	—	+2.05	0.98	0.024	95	+68
Glycine	1500	630	—	—	329	—	—	—	+0.94	0.32	0.003	63	+36
	5000	1017	—	—	427	—	—	—	>1.95	0.36	<0.001	91	+64
DL-Methionine	1500	—	—	574	—	186	—	—	+0.71	0.32	0.017	54	+27
	5000	—	—	783	—	352	—	—	>1.49	0.36	<0.001	81	+54
D Valine	1500	283	257	—	—	—	—	—	+0.18	0.24	0.23	33	+6
	5000	463	646	—	—	—	—	—	>1.07	0.25	<0.001	68	+41
DL Cysteine	1500	—	—	165	—	96	—	—	-0.31	0.24	0.11	18	-9
	5000	—	—	606	—	480	—	—	+1.28	0.46	0.004	75	+48
D Glutamate	1500	93	307	—	—	—	174	55	-0.08	0.21	0.35	24	-3
	5000	217	341	—	—	—	154	36	+0.38	0.22	0.047	41	+14
DL Proline	1500	—	—	386	159	—	254	49	+0.16	0.22	0.24	33	+6
	5000	—	—	695	176	—	412	113	+0.46	0.29	0.062	44	+17
D Leucine	5000	195	194	—	—	—	—	—	-0.10	0.24	0.34	24	-3
DL Lysine	5000	—	—	216	122	—	343	44	-0.13	0.22	0.28	23	-4
D Phenylalanine	5000	272	165	—	—	—	—	—	-0.01	0.23	0.49	27	0
DL Threonine	5000	—	—	—	98	262	314	—	+0.17	0.25	0.26	33	+6

the constituents responsible for germination in the original synthetic medium containing nineteen amino acids. Observations were therefore made (Table 8) with a number of DL and (where available) D amino acids, in a search for further examples of inhibition correlated with stereochemical form. Highly significant inhibitions ($P < 0.02$ for equal or greater effect in the same direction due to chance) were observed both at 1.5 and 5 mM only with glycine and DL methionine. DL-Cysteine and D-valine were

DISCUSSION

The most striking feature of this work is the high specificity of L alanine in promoting germination and the inhibition by its enantiomorph. No such specificity was shown by tyrosine which was replaceable in some degree by related compounds, the carboxyl and unsubstituted amino groups not being essential for activity. The activity of the L form was

not changed significantly when supplied by the synthetic DL-compound

The small molecular ratio of D alanine required to inhibit the action of the L form was especially remarkable since inhibition of microbiological processes by structural analogues usually requires many molecules of inhibitor to one of promoter, e.g. 5000 for sulphonamides and *p* aminobenzoate (Woods, 1940). In the metabolism of amino acids by animal tissues, there are well known examples of the occurrence of inhibitions by enantiomorphs at concentrations comparable to that of the substrate, e.g. synthesis of glutamine (Krebs, 1935), decamination of histidine (Edlbacher, Baur & Becker, 1940). In bacterial chemistry, on the other hand, the use of synthetic DL amino acids as standards in microbiological assay (for review see Snell, 1945a), to avoid active impurities of biological origin, has shown that, in general, only the L forms have been metabolized, the antipodes being inert rather than inhibitory. In some cases the D form may act as a supplement for the L form, but this usually occurs only to a limited extent and requires the presence of sufficient L form to initiate growth (for review see Rydon, 1948). The only species of micro organisms which have so far been shown to be inhibited by D amino acids are *Lactobacillus arabinosus* (*Lb. plantarum*) (Fling & Fox, 1945) and *Escherichia coli* (Kobayashi, Fling & Fox, 1948). Acid production by the former, growing in a synthetic medium, was inhibited by D leucine and D valine at concentrations of the order 200 fold that of the L-forms essential for growth. The latter, growing in nutrient broth, was inhibited 80–90 % by 50 mM D leucine, 80 mM D valine, 120 mM glycine or 140 mM D alanine. *Lb. arabinosus* was also inhibited by glycine at such high concentrations. The present work on germination shows that these amino acids, except D leucine, were inhibitory at 5 mM, as were DL-cysteine and DL methionine also, but D alanine was outstanding by inhibiting even at 5 μ M. The inhibition of growth of *B. anthracis* by unbalanced concentrations of leucine, isoleucine and valine was not due to the D forms (Gladstone, 1939), but with similar inhibitions due to norleucine, serine or threonine the effect of antipodes was not investigated, nor was this done with the inhibition of growth of *Streptococcus lactis* investigated by Snell & Gurard (1943), who also found glycine and β alanine to be inhibitory. With this last named organism, and with *Lb. casei* (Snell, 1945b), D alanine not only failed to inhibit, but at a sufficient concentration, eliminated the need for pyridoxine in growth and in reversing the inhibitions by other amino acids above mentioned.

Even in the case of *B. anthracis*, the inhibition by D alanine was confined to the germination of spores, and growth was not inhibited significantly. Gladstone (1939) grew six strains satisfactorily in an amino acid

medium containing DL alanine, and although the strain used in the present work required supplementation of Gladstone's medium with aneurin, growth was then obtained in the presence of DL alanine, even though the proportion of spores which germinated was not detected by viable counts.

It may be concluded that inhibition by stereoisomers of amino acids is rare in bacteriological chemistry and that elucidation of the reason for this strong inhibition by D alanine, the simplest amino acid capable of showing optical isomerism, might have some bearing on the design of antibacterial agents of therapeutic value (cf Work, 1948). In this connexion, it may be recalled that only the D form of penicillinammonium hydrochloride gave rise to active penicillins on condensation with appropriate oxazolones (quoted by Du Vigneaud, Carpenter, Holley, Livermore & Rachele, 1946). At present no reason can be offered for the importance of the two forms of alanine in promoting and inhibiting germination since existing knowledge of the metabolism of spores (Ruehle, 1923, Cook, 1931, Tarr, 1933, Virtanen & Pulkki, 1933, Keilm & Hartree, 1947) does not include their action on amino acids.

SUMMARY

1 The germination of spores of a virulent strain of *Bacillus anthracis* has been studied by means of viable counts, and the amino acid requirement has been determined.

2 Slow germination occurred in phosphate buffer at pH 7.3, but was greatly stimulated by 500 μ M L alanine, 500 μ M-L tyrosine and 2 μ M adenosine together.

3 L Alanine was not replaceable by related compounds and its action was strongly inhibited by D alanine at a concentration 0.03 times that of its antipode. The inhibition was reversed by increasing the proportion of the L form.

4 L Tyrosine was replaceable by related compounds with little decrease in efficiency, none of the groups—phenolic hydroxyl, amino and carboxyl—being essential for activity. Its activity was not suppressed when supplied as DL-tyrosine.

5 Of seventeen other amino acids tested, none showed significant stimulation of germination. Glycine, DL methionine, DL cysteine and DL valine showed inhibition, but only at concentrations 1000 times that at which D alanine inhibited.

It gives me much pleasure to thank Dr D. W. Henderson and Dr D. D. Woods for careful criticism of this paper. The experimental work was carried out with the technical assistance of Cpl W. Bailey, R.A.M.C., while the author was a member of the Scientific Staff, Medical Research Council. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

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Studies in Vitamin A

14 THE ALLEGED MOBILIZATION OF VITAMIN A BY ADRENALINE

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(Received 13 June 1949)

In 1940 Young & Wald reported that the injection of adrenaline into a rabbit was followed by a very marked increase in the blood vitamin A level, which reached a maximum approximately 30 min after injection. In view of its possible importance in helping to elucidate the mode of action of vitamin A and the possible clinical implications, the effect has been reinvestigated using both rats and rabbits as experimental animals.

EXPERIMENTAL

Animals Rabbits were kept in a constant environment and given an unvarying diet of Lever's cubs and outer leaves of cabbage, rats were maintained similarly except that they were not given cabbage.

Dosing The required dose of adrenaline was injected intraperitoneally into rats and intravenously into a marginal ear vein of rabbits.

Collection and examination of blood The samples of rat blood were withdrawn by cardiac puncture using a method previously described (Glover, Goodwin & Morton, 1946). The samples of rabbit blood (approx 10 ml.) were withdrawn without anaesthesia from a marginal vein of the ear not used for the injection of adrenaline.

In most experiments the plasma was separated from the red cells and examined for vitamin A using the method

developed in this laboratory (Glover *et al.* 1946, Goodwin & Gregory, 1948). In some experiments whole blood was used and extracted according to the method of Young & Wald (1940), but the extract was examined for vitamin A using our method.

RESULTS

Rabbits The results of the rabbit experiments are collected in Table 1. It will be seen that adrenaline has no appreciable effect on the vitamin A plasma levels of rabbit, and an analysis of the data using the *t* test indicated that the differences are not significant.

Young & Wald (1940) used whole blood for their vitamin A tests, but as it is now well established that under normal circumstances no vitamin A exists in the red cells, most of our experiments were carried out on blood plasma. There was, however, a possibility that adrenaline mobilized vitamin A into the red cells and that this would account for Young & Wald's results. To check this, two experiments were carried out in which whole blood instead of plasma was examined for vitamin A, again no mobilization was apparent.

Rats When rats were used the experimental approach had to be altered slightly. The volume of

Table 1 *The action of adrenaline on the blood levels of vitamin A in rabbits*

Animal no	Dose of 1/1000 adrenaline (ml)	Interval between injection and withdrawal of blood (min)	Vitamin A plasma values (i.u./100 ml)	
			Before injection	After injection
C	1.0	120	215	226
120	1.0	30	213	211
120	0.5	30	213	193
134	1.0	10	224	221
81	1.0	35	221	252
113	1.0	10	218	234
161	0.5	30	204	180
167	0.5	30	82	71*
130	0.5	30	55	46*

* The values in these two experiments are those obtained using whole blood instead of plasma only

blood required for each test was more than it was possible to draw from the same animal before and after injection of adrenaline. Consequently, groups of rats (about six per group) of the same sex, age and nutritional background were used, one group was injected with adrenaline and the other served as a control. The vitamin A plasma level of each rat was determined separately and the mean values for the two groups compared. It will be seen from Table 2 that no significant differences could be demonstrated between the control groups and those treated with

of vitamin A (Glover *et al.* 1946), thus any substance which can mobilize vitamin A alcohol into the blood from the mainly esterified liver reserves might be of considerable importance clinically. Clausen, Baum, McCoord, Rydeen & Breese (1940) claimed that ethanol mobilized vitamin A in dogs and later the same group of workers (Clausen, Breese, Baum, McCoord & Rydeen, 1941) stated that drinking liquor to the extent of 'the usual "social" evening common in this country' (U.S.A.) raised the vitamin A plasma levels of humans. Brenner & Roberts (1943) also found a rise in humans. However, not only have British investigators failed to observe any rise after administration of ethanol (Yudkin, 1941; Hume & Krebs, 1949), but the increase recorded by the American investigators was almost entirely due to esterified vitamin A (Clausen, Baum, McCoord, Rydeen & Breese, 1942), so that any such rise would apparently serve no useful therapeutic purpose. This raised the question whether the surprisingly large increase in the vitamin A level of plasma after adrenaline injections reported by Young & Wald (1940)—a doubling within 30 min—was in the alcohol or the ester fraction.

We could not test this point, however, as we were unable to reproduce Young & Wald's results, no mobilization of vitamin A was apparent in either plasma or red cells. It is impossible at the moment to offer any explanation of these contradictory results.

Table 2 *The action of adrenaline on the plasma levels of vitamin A in rats*

Exp. no	Dose of 1/1000 adrenaline (ml/rat)	No. of rats		Interval between injection and withdrawal of blood (min)	Mean vitamin A plasma levels (i.u./100 ml.)	
		Control group	Test group		Control group	Test group
1	0.5	7	7	30	49.9	49.8
2	0.5	10	3	60	35.8	40.4
3	0.5	8	4	15	38.0	45.7
4	0.5	4	4	14	61.7 64.3 66.3 57.1	61.7* 66.8 68.8 61.7
					Mean 62.3	64.7

* The results of Exp. 4 are recorded in detail to illustrate the usual variations found between plasma levels of rats of the same group.

adrenaline. It should be noted that the variations between the values obtained on rats in a single group are quite small, and could thus in no way mask an effect as large as that encountered by Young & Wald (1940), to illustrate this the individual results are quoted for Exp. 4 (Table 2).

DISCUSSION

Owing to the existence of a blood liver equilibrium the plasma levels of vitamin A alcohol (the functional form) are only slightly raised even after large doses

SUMMARY

1 The claim that intravenous injection of adrenaline into rabbits mobilizes liver vitamin A into the blood has not been confirmed.

2 Similar negative results were obtained with rats.

This work was carried out whilst one of us (A.A.W.) was in receipt of an Animal Health Trust Fellowship. The investigations of which this forms a part have been assisted by the Ministry of Food and the Medical Research Council.

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FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

- The intracellular mode of action of the sulphonamide derivatives. Some condensation products of reductone. By E A BELL, WESLEY COCKER and R A Q O'MEARA
- The micro-estimation of citric acid. By H. WEIL-MALHERBE and A D BONE
- Studies in detoxication. 27 The orientation of conjugation in the metabolites of 4 chlorocatechol and 4-chlororesorcinol, with some observations on the fate of (+) adrenaline, protocatechuic acid and protocatechuic aldehyde in the rabbit. By K. S DODGSON and R T WILLIAMS
- Occurrence of cytochrome and coproporphyrin in *Mycobacteria*. By C M TODD
- Quantitative studies on mutants of *Bacterium aerogenes* requiring nicotinic acid and histidine. By PULLAKOTE DEVI and CONSTANCE HIGGINBOTTOM
- The composition of foetal fluids of sheep at different stages of gestation. By E I McDUGAL
- A photoelectric flame photometer. By W R DOMINGO and W KLYNE
- Vitamin A in seals. By K. RODAHL and A W DAVIES
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- Long chain unsaturated fatty acids as essential bacterial growth factors. Substances able to replace oleic acid for the growth of *Corynebacterium* 'Q' with a note on a possible method for their microbiological assay. By M. R POLLOCK, G A HOWARD and B W BOUGHTON
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- The biochemistry of locusts. 2 Carotenoid distribution in solitary and gregarious phases of the African migratory locust (*Locusta migratoria migratoroides*, R & F) and the desert locust (*Schistocerca gregaria*, Forsk.) By T W GOODWIN
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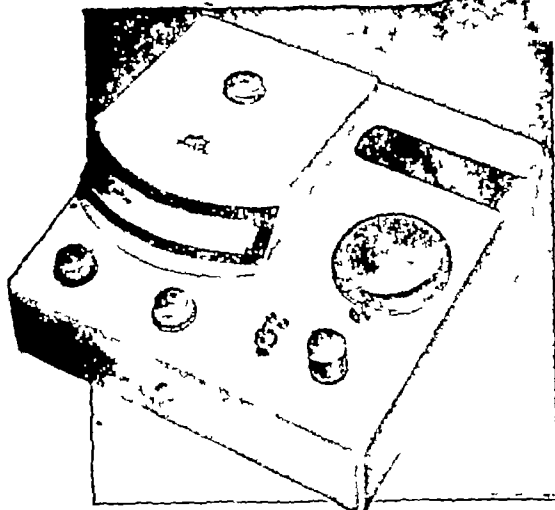
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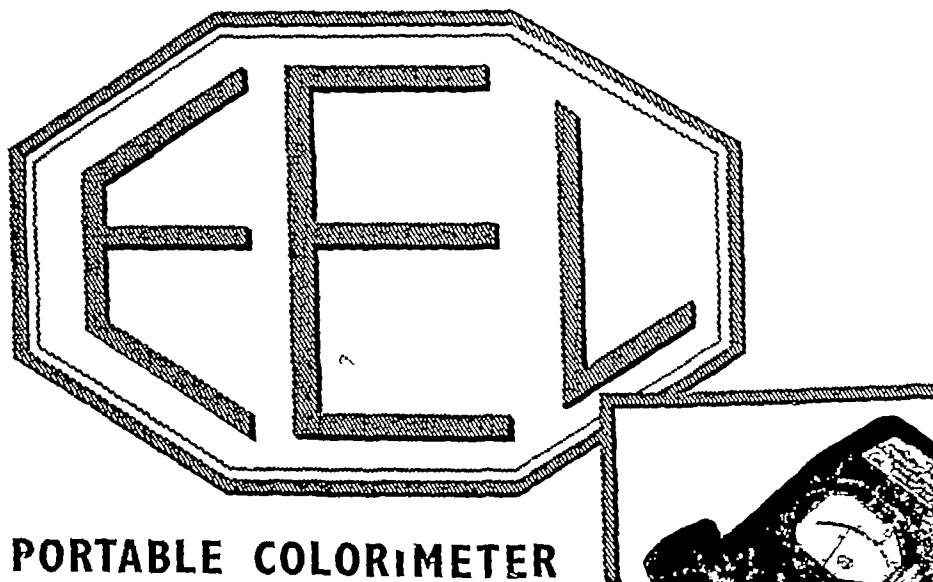
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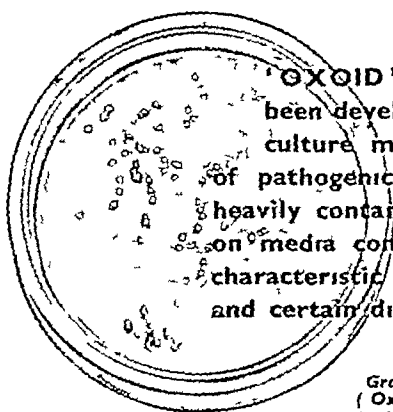
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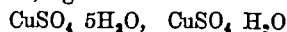
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CORRIGENDA

The Estimation of a Fraction Resembling Pregnanediol Glucuronide in Human Urine

By N G Bisset, B W L Brooksbank and G A D Haslewood

Volume 42 (1948), No 3, p 368, col 2

line 51 *for* $\text{Na}_2\text{HPO}_4(0.05 \text{ M})$
 read $\text{Na}_2\text{HPO}_4(0.2 \text{ M})$

Volume 48 (1948), No 4, Contents p ii

line 1 *for* 2
 read 3

Studies on Cholinesterase 6 The Selective Inhibition of
True Cholinesterase *in Vivo*

By Rosemary D Hawkins and B Mendel

Volume 44 (1949), No 3, p 261, col 2

line 16 *for* 3004
 read 300 4

The Intermediary Metabolism of the Mammary Gland

1 RESPIRATION OF LACTATING MAMMARY GLAND SLICES IN PRESENCE OF CARBOHYDRATES

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(Received 3 February 1949)

Studies of the respiratory exchange of the mammary gland have hitherto been confined to ruminants in which the arterio venous method can conveniently be applied to the udder. Though blood velocity data, which are difficult to obtain, are necessary to convert arterio venous differences in oxygen and carbon dioxide into absolute values for uptake and output respectively, the respiratory quotient (RQ) can be obtained without blood flow measurements.

Results in the goat (Graham, Houchin, Peterson & Turner, 1938, Reineke, Stonecipher & Turner, 1941), and cow (Petersen & Shaw, 1942, Shaw, 1946) point to an RQ greater than unity for the lactating udder, which has been interpreted (Graham *et al* 1938, Reineke, Stonecipher & Turner, 1941) as indicating the formation of milk fat, more specifically of the short chain fatty acids (Reineke, Stonecipher & Turner, 1941), from carbohydrate.

In many of the above series of determinations, at any rate those on non anaesthetized animals, the individual values were rather variable, and indeed the high variance of their results occasioned comment by Petersen & Shaw (1942). However, a small series of determinations on cows in this laboratory (Bottomley & Folley, unpublished results), in which the blood samples were protected against gas exchange during and after collection by use of the apparatus of Austin, Cullen, Hastings, McLean, Peters & Van Slyke (1922), were satisfactorily uniform, giving a mean $RQ = 1.25 \pm 0.12$ (95% fiducial limits) in cases where no haemoconcentration, indicative of manipulative disturbance of the subject, occurred during blood sampling. When significant haemoconcentration occurred the RQ was usually less than unity.

Working on anaesthetized goats, to minimize the risk of disturbance during blood sampling, Reineke, Stonecipher & Turner (1941) obtained a fairly uniform series of values, but the mean RQ (1.09) could hardly be considered significantly greater than unity, though it was raised to 1.18 by applying, following the precedent of Graham *et al* (1938), a correction for CO_2 believed to be used for the synthesis of urea by the udder. As regards anaesthetized cows, a series of values obtained by Shaw (1946), which gave a mean RQ of 1.27, was perhaps not as uniform as would be desirable.

In sum it might fairly be said that while there is a certain amount of evidence suggesting a high RQ for the lactating ruminant udder *in vivo*, it is by no means as clear cut and conclusive as might be desired, particularly in view of criticisms which have been levelled against the arterio venous technique as applied to the udder (Kay, 1947, Folley, 1949). Because of this, and because RQ values reported for the perfused isolated bovine udder have been less than unity (Shaw, 1939) or hardly significantly greater (Petersen & Shaw, 1942), an investigation of the respiratory metabolism of the lactating mammary gland by the tissue slice method appeared to be of interest. This was the more so because, although mammary gland slices have been used for studying lactose formation *in vitro* (Grant, 1935, 1936, Knodt & Petersen, 1945), the only *in vitro* study of the respiration of mammary tissue known to us is that of Kleiber, Smith & Levy (1943), who used 'portions' of mammary tissue which evidently were not slices in the accepted sense, and who did not determine RQ . The tissue slice method has the advantages that it is applicable to all mammals, thus enabling the ruminant udder to be compared with the mamma of the non ruminant, and it can be used for studies of phases of intermediary metabolism which are hardly open to attack by the arterio venous method. The results of an investigation of the respiration of mammary gland slices from various species, metabolizing carbohydrates, are given in the present paper. Part of this work has already been published in preliminary form (Folley & French, 1948a, b).

METHODS

Animals. Most of the experiments were carried out on mammary tissue from rats, two strains of which were used, Wistar and hooded Norway. The majority were autopsied at the height of lactation (15th–21st days) as were also most of the mice (Swiss strain) used. Groups of rabbits (Dutch) were killed on the 7th and 25th–28th days of lactation and guinea pigs on the 10th–12th days. The animals were killed by dislocation of the spinal cord and mammary tissue immediately removed, the litters being allowed to suckle right up to the time of death.

Udder tissue was obtained from lactating goats, mostly crossbreeds bought in, but in a few cases from pedigree British Saanens of the Institute herd. Usually the goats were shot

and the tissue removed immediately, in three cases pieces of tissue for slicing were removed from living goats under cyclopropane anaesthesia. The acquisition of authentic lactating udder tissue from cows giving adequate yields of milk presents much more formidable difficulties since cows are not normally slaughtered while in milk. We were, however, able to obtain tissue from a few cows culled from the herds of the Institute or the University of Reading, four of them giving about 10 lb of milk daily and all milked regularly up to slaughter.

Mammary gland slices In the case of rats, mice and rabbits, abdominal* mammary glands were taken, except in one experiment on rats (see p. 119) in which only thoracic glands were available. Because of the structure of mammary tissue, which consists of lobules of alveoli bounded by septa of tough, elastic connective tissue, suitable thin slices of lactating mammary gland are difficult to cut by the usual methods. It was, however, possible to cut satisfactory slices less than 0.4 mm thick by means of the microtome of Stadie & Riggs (1944). On removal from the microtome the slices, if straightened out by forceps on a flat surface, are seen to be of such irregular, lacy form, consisting of small pieces (presumably lobules) of alveolar tissue attached to a network of connective strands, that the area cannot be measured, thus precluding determination of thickness by the usual method. The more or less constant slice thickness given by the microtome was therefore determined as usual on liver slices cut under similar conditions. A slice thickness of 0.4 mm or less seems to be necessary for work with mammary gland since slices about 0.6 mm thick give a lower Q_{O_2} .

Quotients (Q_{O_2} , Q_{CO_2} , Q_{acid}) were calculated in $\mu\text{l}/\text{mg}$ final dry wt./hr., they were not calculated on the basis of initial dry weights, as recommended by many workers, because lactating mammary tissue retains considerable quantities of milk in the alveoli (Folley & Greenbaum, 1947). Milk is leached out from the slice during a manometric experiment, as evidenced by the increasing opacity of the medium, and although no attempt has been made to determine the final milk content it seems unlikely that much would remain in a thin slice under these conditions. The final dry weights of the slices were thus used for the calculation of the quotients, and errors due to the presence of small amounts of milk at the end of the experiments would tend to compensate for those due to loss of tissue fragments during shaking. In actual fact, mammary gland slices, though looking rather fragile and indeed tattered, did not show any tendency to break up during the experiments.

Cow udder slices were cut at the abattoir, placed in Ringer bicarbonate (gassed with 95% O_2 , 5% CO_2) at room temperature and at once brought to the laboratory. The extra delay from animal to manometric flask was about 10 min.

Duplicate determinations on different slices from the same piece of tissue mostly showed satisfactory agreement, though occasionally there were larger differences (e.g. Fig. 3), probably due to the presence of different proportions of parenchyma and stroma in the slices. The presence of non-secretory tissue, as well as the above mentioned 'milk error',

would not affect πQ , but would cause some underestimation of other quotients.

Manometric methods All experiments were done at 37° πQ , Q_{O_2} and $Q_{acid}^{0.1}$ were determined by the method of Dickens & Šimer (1931a) over 3 hr using Dickens & Greville (1933b) flasks. The medium was the Ringer bicarbonate of Krebs & Henseleit (1932) and the gas mixture 95% O_2 , 5% CO_2 . In some experiments O_2 uptake was measured in Ringer phosphate using ordinary Warburg flasks and pure O_2 . For determinations of anaerobic acid production the gas mixture was 95% N_2 , 5% CO_2 passed over hot Cu foil. The Warburg vessels contained 2.5 ml of Ringer bicarbonate and were gassed for 15 min. before the taps were closed.

With few exceptions determinations were in duplicate. In most experiments one or two slices (total 80–100 mg) were used, but in the case of bovine udder tissue it was necessary to use more tissue to get a sufficient rate of gas exchange.

RESULTS

Rate of oxygen uptake

Typical experiments on gland from a fully lactating rat are shown in Figs. 1 and 2. The tissue shows an appreciable O_2 uptake in Ringer phosphate (pH 7.4) in absence of substrate, and the rate falls off gradually over 3 hr. With glucose (0.2%) present from the outset the respiration rate was much higher and linear over 3 hr (Fig. 1), while if the glucose was added from a side arm after 60 min. the tissue could still respond with an unmistakable enhancement in uptake (Fig. 2). The marked effect of glucose on the respiration rate of this tissue is in accord with the relative lack of reserve carbohydrate, as evidenced by our unpublished observations of the low glycogen content of rat mammary gland (see Folley, 1949) and by the non utilization of lactose (see p. 120).

Respiration of rat mammary gland in presence of glucose

The results obtained with rat mammary gland in the presence of glucose are summarized in Table 1. The fully lactating gland, i.e. at 15–21 days of lactation under laboratory conditions (see below), shows in the presence of glucose a fairly high Q_{O_2} (9–10) which is almost double the endogenous value. Analysis of data obtained at different stages of lactation, mostly to be presented in a subsequent paper, indicated no great change in Q_{O_2} over the period 15–21 days, so that data for rats killed within this period have been pooled throughout.

The question of the amount of substrate needed for 3 hr experiments on mammary tissue needs consideration, because this tissue may utilize glucose for synthesis of lactose, and possibly fat, as well as for oxidation. The effect of glucose concentrations varying from 0.1 to 1.0% was therefore investigated in a series of experiments in which the effect of each glucose concentration was compared with that of 0.2%, the standard concentration adopted at the

* In this paper the word 'abdominal' as applied to mammary glands refers to the two abdominal and four inguinal glands.

outset of this work, on duplicate slices from the same animal. There was no evidence that glucose concentrations greater than 0.2% were more effective in increasing the total respiration over 3 hr (see Table 1 for results with 0.2 and 0.3% glucose respectively), but in order to allow an ample margin, 0.3% glucose was adopted for the later experiments. Results with 0.2 and 0.3% glucose (or other carbohydrate) were pooled whenever necessary in compiling the Tables given in this paper.

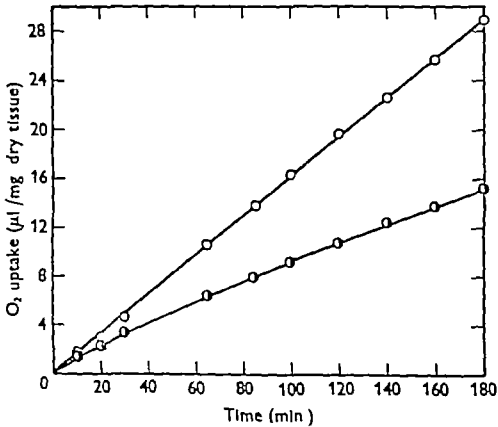


Fig 1 Oxygen uptake of mammary gland slices from rat on nineteenth day of lactation. Upper curve 0.2% glucose present from the outset, lower curve no substrate. Temp 37°, pH 7.4, phosphate buffer, atmosphere, 100% O₂.

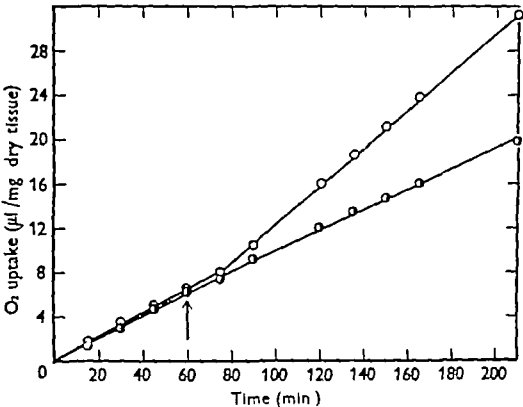


Fig 2 Oxygen uptake of mammary gland slices from rat on nineteenth day of lactation. Upper curve 0.2% glucose added from side arm at arrow, lower curve no substrate. Temp 37°, pH 7.4, phosphate buffer, atmosphere, 100% O₂.

Table 1 includes results for a group of rats in which slices were cut from thoracic glands because the abdominal glands were required for another purpose. We consider the thoracic glands to be less suitable for metabolic work than the abdominal glands because they tend to be less suckled, and therefore might lactate less actively, and because they are interlaced with strands of muscle tissue. Nevertheless, the mean Q_{O_2} was, if anything, slightly higher than for abdominal glands (Table 1).

Combining the data for three groups of rats killed at 15–21 days (Table 1), the mean $-Q_{O_2}$ for thirty-

lactation, the mean value for the thirty nine rats killed at 15–21 days being 1.53 ± 0.02 (see Table 2). This happens to be double the value (0.77 ± 0.03 , for ten rats) obtained in the absence of substrate (Table 1). Lasar (1942) has criticized the validity of RQ determinations on tissue slices over periods as long as 3 hr. However, there seems to be no reason to doubt the accuracy of our values on these grounds, since in experiments with rat mammary gland slices in which the curve expressing the resultant of O₂ uptake and CO₂ output (the 'composite curve' of Ellhott, Greig & Benoy, 1937) was studied over 3 hr,

Table 1 Respiration of lactating rat mammary gland slices in presence of glucose

No of rats	Stage of lactation at autopsy (days)	Substrate	$-Q_{O_2}$ (μl/mg final dry wt/hr)		RQ	
			Mean*	Range	Mean	Range
2	6 and 12	Nil	—	4.5, 6.5	—	0.73, 0.68
10	15–21	Nil	5.3 ± 0.3	4.1–7.8	0.77 ± 0.03	0.68–0.92
3	3–6	Glucose (0.2%)	5.6	3.6–7.7	1.47	1.37–1.66
20	15–21	Glucose (0.2%)	9.1 ± 0.3	6.8–11.5	1.53 ± 0.03	1.31–1.86
8†	17	Glucose (0.2%)	9.9 ± 0.4	7.8–11.2	1.51 ± 0.03	1.33–1.67
11	15–21	Glucose (0.3%)	9.3 ± 0.4	6.3–11.9	1.55 ± 0.03	1.41–1.73
3	22–25	Glucose (0.2 or 0.3%)	9.3	6.9–11.1	1.29	1.11–1.44

* In this and subsequent Tables results are shown as mean ± S.E. where appropriate.
† Slices from thoracic glands, all others from abdominal glands.

CO₂ output exceeded O₂ uptake from the outset, and the curves were linear over most of the period only showing a slight falling off towards the end. This latter point would indicate that our R.Q.'s were if anything underestimated.

Respiration of rat mammary gland slices in presence of various sugars

The respiration of mammary gland slices from rats killed at 15–21 days of lactation was investigated in the presence of a number of carbohydrates. In all experiments substrate concentrations were 0.2 or 0.3%, except in the case of lactose which was used in 0.4 or 0.6% concentration. The results are given in Table 2.

Of the sugars studied, which include those known to be of outstanding importance in mammalian biochemistry, mannose was the only one besides glucose

in the rat, some representatives of each form (mouse, guinea pig, rabbit) were taken early in lactation, but after milk secretion could be considered as well established, while others were taken some days later (see Table 3) but, it was believed, before lactation began to decline because of gradual self weaning of the young. In the case of the mouse, the second group were killed at 15 days by analogy with the rat and it seems probable that these glands were in full lactation. The guinea pig presented most difficulty because its young are capable of fending for themselves at birth so that the demands on, and functional response of, the mammary gland in the guinea pig under laboratory conditions are quite problematical.

For the goats, most of which were purchased (in milk), the stages of lactation were unknown save in four cases, all of which were in fairly advanced lactation. Since lactating goats bought in the autumn, as these were, are most likely to have kidded during the previous February or March, the results may be taken as typical for the udder of the goat after eight or more months' lactation. With three of the four goats

Table 2 *Respiration of lactating rat mammary gland slices in presence of various carbohydrates*

(All rats killed between 15 and 21 days of lactation and all slices taken from abdominal glands except in eight of the experiments with glucose in which thoracic tissue was used. Substrate concentrations 0.2 or 0.3%, except for lactose which was 0.4 or 0.6%.)

Substrate	No. of expts	-Q _{O₂}		R.Q.	
		Absolute (μl/mg final dry wt/hr)	Percentage of value given by another slice from the same animal in presence of glucose	Absolute	Percentage of value given by another slice from the same animal in presence of glucose
D Glucose	39	93 ± 0.2	—	153 ± 0.02	—
D Galactose	3	6.3	61	0.78	58
D Mannose	4	9.7	96	1.55	102
D Fructose	3	5.9	71	0.78	53
L Sorbose	1	6.8	69	0.63	39
D Glucose 1 phosphate	1	7.2	59	0.96	69
N Acetylglucosamine	1	4.4	44	0.89	57
Glucosamine	1	6.4	62	0.84	53
L-Arabinose	1	6.9	68	0.68	46
D Arabinose	1	6.0	48	0.78	46
D Ribose	1	4.9	53	0.72	42
D Xylose	1	4.5	36	0.77	46
L-Rhamnose	1	6.0	70	0.80	50
Lactose	3	5.4	65	0.79	57
Maltose	1	6.5	80	0.85	57
Cellobiose	1	5.8	72	0.73	49
Sucrose	1	5.5	53	0.82	52
Raffinose	1	4.1	48	0.88	55
Glycogen	1	6.7	62	0.70	43

to be utilized by lactating mammary gland slices, as evidenced by a Q_{O₂} equal to that shown in the presence of glucose and by an R.Q. greater than unity. In a few cases (lactose, fructose, sorbose), in which the endogenous respiration was studied on duplicate slices, Q_{O₂} was not increased in presence of the sugar.

Respiration of mammary tissue of various species in presence of glucose

In studying the respiration of lactating mammary tissue from various species the question arose when to kill the animals so as to obtain fully lactating tissue. Since the course of lactation in the other common laboratory mammals, with the possible exception of the mouse, is less well known than

(pedigree animals from the Institute herd) whose kidding dates were known, mammary tissue was removed under cyclopropane anaesthesia. The results for these are given separately in Table 3, although there is no evidence that the anaesthetic affected the issue. Of the six cows available, all save two were of necessity in rather advanced lactation, although four were yielding approximately 10 lb milk/day at slaughter. In all cases the substrate was 0.3% glucose except for some rats where 0.2% was used.

The results are summarized in Table 3, which indicates that Q_{O₂} is inversely correlated with body size, mouse being the most active tissue studied (-Q_{O₂} = 16.0) and cow the least (-Q_{O₂} = 3.5). As with the rat, R.Q. was significantly greater than unity in

Table 3 *Respiration of lactating mammary gland slices from various species in presence of glucose**

Animal	Stage of lactation	No of animals	$-Q_{O_2}$ (μ l/mg final dry wt /hr)		R.Q	
			Mean	Range	Mean	Range
Mouse	5 days	3	10.5	8.3-12.0	1.65	1.52-1.80
	15 days	6	16.0 \pm 1.0	13.3-19.0	1.94 \pm 0.07	1.66-2.11
Rat	15-21 days	39	9.3 \pm 0.2	6.3-11.9	1.53 \pm 0.02	1.31-1.86
Guinea pig	5 days	1	9.6	—	1.06	—
	10-12 days	5	9.0 \pm 0.4	8.3-10.0	1.17 \pm 0.06	1.01-1.32
Rabbit	7 days	6	6.6 \pm 0.3	5.2-7.2	1.45 \pm 0.05	1.28-1.60
	25-28 days	3	5.8	3.6-8.2	1.32	1.21-1.52
Goat	Unknown	8	4.6 \pm 0.3	3.2-5.7	0.86 \pm 0.03	0.69-0.95
	Advanced†	3	5.7	5.0-6.2	0.85	0.74-0.93
Cow	Milk yield approx. 10 lb /day	4	3.5 \pm 0.5	2.2-4.7	0.80 \pm 0.04	0.64-0.93
	Milk yield < 5 lb /day	2	—	1.6, 1.9	—	0.87, 0.90

* Glucose concentration 0.3% except in the case of some rats where 0.2% was used

† Tissue removed under anaesthesia

Table 4 *Acid production by lactating mammary gland slices*

Animal	Stage of lactation (days)	Substrate	No of exps	Q_{acid} (μ l /mg final dry wt /hr)	
				Mean	Range
Aerobic*					
Mouse	15	Glucose (0.3%)	6	1.7 \pm 0.3	0.9-2.4
Rat	15-21	Nil	10	0.6 \pm 0.1	0.2-1.0
Rat	15-21	Glucose (0.2 or 0.3%)	39	1.7 \pm 0.1	0.8-2.9
Guinea pig	10-12	Glucose (0.3%)	5	0.7 \pm 0.1	0.3-0.9
Rabbit	7	Glucose (0.3%)	6	1.4 \pm 0.3	0.7-2.9
Goat	Unknown	Glucose (0.3%)	8	1.6 \pm 0.1	1.2-2.2
Cow	Various stages	Glucose (0.3%)	6	1.7 \pm 0.2	1.0-2.3
Anaerobic†					
Rat	15-21	Glucose (0.3%)	6	9.6 \pm 1.3	7.3-13.8

* Calculated over 3 hr

† Calculated over initial 30 min

the mouse, guinea pig and rabbit, but it was less than unity in the ruminants, cow and goat. Of the non-ruminants, mouse tissue gave the highest R.Q., the value being not significantly less than 2.

Acid production of lactating mammary gland slices

Values for the aerobic acid production obtained in 3 hr experiments on tissue from various species are given in Table 4. Under aerobic conditions in bicarbonate medium the acid production is not unduly high by comparison with other tissues (Dickens & Simer, 1931b; Dickens & Greville, 1933a), and there are no prominent species differences. The actual glycolysis may be even lower, since part at least of the acid production of mammary tissue may be due to formation of citric acid (Knodt & Petersen, 1946) or to synthesis of fatty acids.

Anaerobic experiments were carried out on rat tissue only. Typical experiments in which the rate of anaerobic acid production over 3 hr periods was studied are illustrated in Fig. 3. The initially high rate of acid production falls off steadily over the whole period, hence the values for $Q_{CO_2}^N$, given in Table 4, were calculated from data for the initial 30 min during which the rate of acid production is nearly linear. The reason for this steady falling off in acid production under anaerobic conditions is not known, exhaustion of the substrate was not responsible because the addition of extra glucose did not prevent it. It was not due to tissue damage caused by lowering of the pH of the medium, since in the experiment in which most acid was produced, the pH only fell by 0.2 unit over 3 hr. As in the case of aerobic acid production, it is possible that not all of the acid produced under anaerobic conditions is due to glycolysis. Nevertheless, it seems safe to

conclude from these results that mammary tissue exhibits a considerable Pasteur effect. The curves in Fig 3 also illustrate the variation to be expected between duplicate slices from the same rat, and the lowered acid production in absence of substrate

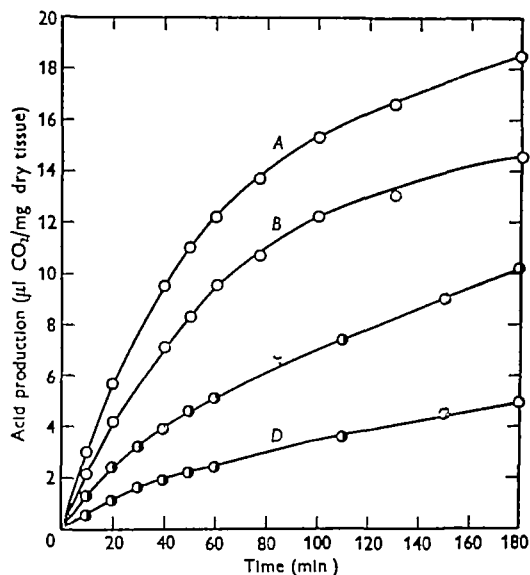


Fig 3 Anaerobic acid production of slices of lactating rat mammary gland. Curves A and B relate to duplicate slices from a rat on the fifteenth day of lactation, both in presence of 0.3% glucose. Curves C and D relate to duplicate slices from a rat on the twenty first day of lactation, C with 0.3% glucose, D with no substrate. Temp 37°, pH 7.4, Ringer bicarbonate, atmosphere, 95% N₂, 5% CO₂.

Effect of reduced food intake and adrenalectomy on the respiration of mammary tissue in the rat

The relation between the intensity of lactation and the respiratory metabolism of mammary tissue is of interest, and it was felt that a study of procedures which depress lactation, such as underfeeding or adrenalectomy, might provide preliminary informa-

tion in this connexion. Thoracic mammary tissue was available for study from rats which had been adrenalectomized on the fourth day of lactation and also from sham operated rats pair fed to the reduced food intake of these, as well as from sham operated rats fed *ad lib*. Details of the diet and general experimental procedure were as described for a similar experiment by Cowie & Folley (1948). All rats were killed on the seventeenth day of lactation and the slices were incubated with 0.2% glucose.

The results are given in Table 5. Lactation was depressed in both adrenalectomized and pair fed sham operated rats, markedly in the former and less so in the latter, as indicated by the litter growth indices (Cowie & Folley, 1947). It is important to note that despite this the adrenalectomized rats and the pair fed controls were still suckling young at the time of autopsy. Q_{O_2} was significantly depressed, and, notwithstanding the difference in lactational performance, to about the same extent in tissue from the adrenalectomized rats and from the pair fed controls, while on the other hand, the aerobic acid production was significantly raised. Particularly striking is the fact that in tissue from both these groups the R:Q was reduced to a value not far from unity. The possibility that, since the experiment was relatively long term, the mammae of the two groups under consideration had undergone some degree of involution by the time of autopsy must obviously be considered. However, Folley & Greenbaum (1948), in a discussion of the effects of these procedures on the mammary gland arginase levels, concluded that this was unlikely.

The results indicate that a period of partial inanition significantly lowers the Q_{O_2} of lactating mammary tissue, increases the apparent aerobic glycolysis and also depresses the high R:Q to values near unity, i.e. to values intermediate between those given by fully lactating tissue in presence and absence of glucose respectively. Moreover, it would appear that adrenalectomy *per se* has no effect on the respiratory metabolism of the mammary gland.

Table 5 Respiration and apparent aerobic glycolysis of slices of lactating rat mammary gland from adrenalectomized and pair fed rats

(All rats operated upon on the fourth day of lactation and killed on the seventeenth day. Respiration measurements made at 37° in presence of 0.2% glucose in Ringer bicarbonate, pH 7.4.)

Treatment	No of rats	Mean food intake from 4 to 17 days (g/day)	Litter growth index*	$-Q_{O_2}$ (μl/mg final dry wt/hr)		R:Q		Q_{acid}^0 (μl/mg final dry wt/hr)	
				Mean	Range	Mean	Range	Mean	Range
Sham operation fed <i>ad lib</i>	8	36.7 ± 1.02	13.0	9.9 ± 0.4	7.8-11.2	1.51 ± 0.03	1.33-1.67	1.8 ± 0.2	1.2-2.6
Sham operation pair fed	9	24.1 ± 0.65	11.2	8.0 ± 0.3	6.0-8.9	1.05 ± 0.04	0.85-1.24	4.4 ± 0.4	2.3-6.9
Adrenalectomy on fourth day of lactation fed <i>ad lib</i>	9		6.1	7.6 ± 0.3	5.7-8.8	0.95 ± 0.06	0.70-1.24	3.0 ± 0.3	1.7-4.5

* See Cowie & Folley (1947)

DISCUSSION

The results presented in this paper show that, as might be expected from its outstanding synthetic activity, lactating mammary tissue respire fairly actively *in vitro* by comparison with other tissues. In the rat its activity is much less than that of nervous tissues or kidney cortex, but is of the same order as liver (Dickens & Šmer, 1931*b*, Dickens & Greville, 1933*a*). Our Q_{O_2} values for the rat are considerably higher than those given by Kleiber *et al* (1943), but since these workers did not use thin slices it seems possible that their tissue was not equilibrated with O_2 . Unlike some tissues, for example liver and kidney, the reserves of oxidizable substrates present in mammary tissue are low, since Q_{O_2} is markedly increased over endogenous values in the presence of glucose, but the effect is not comparable with that shown by nervous tissues (Dickens & Greville, 1933*a*). The low RQ obtained in the absence of substrate suggests that under these conditions the slice is oxidizing fat and perhaps protein. An interesting inverse correlation between Q_{O_2} for mammary tissue and body size, among various animal forms, is clearly indicated by our results.

The anaerobic acid production of rat mammary gland is quite considerable, though not as high as nervous and embryonic tissues (Dickens & Greville, 1933*a*), but since mammary tissue may produce citric, and under some conditions, fatty acids, it is doubtful whether the acid production measured by us either in the presence or absence of O_2 is wholly ascribable to glycolysis. For that reason we have not considered it worth while to estimate the Pasteur effect quantitatively, but it seems safe to conclude from our results that it is considerable.

Our results serve to complicate somewhat the position regarding the mammary gland RQ . The *in vivo* experiments, mentioned earlier, which indicated an RQ greater than unity for the lactating mammary gland, were of necessity performed on ruminants (cow and goat). In our *in vitro* experiments, while RQ values considerably greater than unity have been given by mammary tissue from the mouse, rat, guinea pig and rabbit, all experiments with tissue from the cow and goat indicate an RQ less than unity with glucose as substrate. It may be objected that our ruminant slices were taken from udders which had been in lactation for considerable periods, and which might in consequence have contained considerable proportions of cells not in a state of optimal functional activity. Against this, it may be stated that slices from many of the same udders (cow and goat) have given RQ 's greater than unity in presence of acetate (Folley & French, 1948*c*, 1949), a product of rumen fermentation which is almost certainly of great importance as a metabolite in the ruminant (Elsden & Phillipson, 1948).

The high RQ indicated by *in vivo* experiments for the ruminant udder has been interpreted (Graham *et al* 1938, Reineke, Stonecipher & Turner, 1941) as pointing to the synthesis of fat from carbohydrate in the active mammary gland. While our *in vitro* determinations lend no support to this theory as far as the ruminant is concerned, we feel that, despite the need for caution in interpreting the RQ of even a single organ (e.g. Soskin, 1941), our results with mammary gland slices from non ruminants strongly suggest the possibility of fat synthesis from carbohydrate in these non ruminant glands. Indeed, the possibilities of using rat or mouse mammary gland, especially the latter in view of its exceptionally high RQ , for *in vitro* studies of fat synthesis seem particularly attractive, since mammary gland seems to be practically unique among mammalian tissues in giving a high RQ in the presence of glucose *in vitro*, coupled with a considerable respiration and hence, presumably, an appreciable rate of synthesis. A high RQ has been reported for adipose tissue (Henle & Szpingier, 1936, Mirski, 1942), but the rate of gas exchange is so low as almost to preclude accurate measurement. As for the high *in vivo* RQ of the ruminant udder, we have pointed out (Folley & French, 1949) that it is consistent with the synthesis of fat from acetate, a process which ruminant udder slices appear capable of effecting *in vitro*.

The striking respiratory response of mammary gland slices to mannose as well as glucose, while other sugars are inert, may be of significance in connexion with the mechanism of lactose synthesis. Reineke, Williamson & Turner (1941) have reported a significant uptake of blood glycoprotein by the udder of the lactating goat. Blood glycoproteins are known to contain mannose which, our results suggest, would be available for any purpose for which the gland uses glucose. However, Grant (1935) found no evidence of synthesis of lactose from mannose by guinea pig mammary gland slices, which he reported were capable of effecting the synthesis from glucose. Nevertheless, in view of recent evidence (Friedmann, 1949) that, aside from glucosamine, the carbohydrate moiety of serum glycoproteins consists of an equimolecular mannose galactose complex, the question whether lactose, or part of it, is formed in the mammary gland by transformation of a mannose galactoside arising from blood protein seems worth consideration, particularly since our results suggest that glucose and mannose are interconvertible in the mammary gland. The non utilization of lactose, hardly surprising since the mammary gland would not be expected to secrete a carbohydrate which it could readily oxidize, indicates that the chain of reactions from glucose to lactose is not reversible, at any rate at an appreciable speed, under these conditions. Folley (1949), in proposing a possible scheme for the biological synthesis of lactose, has discussed

the possibility that the final step may be an irreversible dephosphorylation. The failure of the slices to oxidize galactose and fructose indicates that these sugars are not convertible to glucose by this tissue as they are by the liver. In respect of attack on fructose, mammary gland differs from brain, retina, testis and kidney, all of which oxidize fructose (Dickens & Greville, 1933*a*). The non-utilization of glucose 1 phosphate may be due, as suggested by Grant (1936), to the inability of sugar phosphates to penetrate into the mammary gland cell.

The effects of long continued partial manition on the lactating mammary gland, which are overtly manifested by a reduction in milk yield, are also reflected in a reduction of the respiration rate and by a fall in the RQ to values near unity. This no doubt means that the synthesis of fat from oxygen rich compounds is preferentially decreased under these conditions. The effects of adrenalectomy, which also causes a depression of milk yield over and above that ascribable to post operative anorexia (Cowie & Folley, 1948), could not be quantitatively distinguished from those due to post operative anorexia. In this respect the enzyme systems responsible for the respiratory metabolism differ from arginase which is believed to play an important role in mammary metabolism in the rat (Folley & Greenbaum, 1947), for the arginase levels of the rat mammary gland were decreased by adrenalectomy but not by partial manition (Folley & Greenbaum, 1948). The damage to the function of the mammary cells due to these two procedures is associated not only with a decreased respiration, but also by a significant increase in the apparent aerobic glycolysis. Similar effects following weaning will be described in a subsequent paper.

SUMMARY

1 The respiratory metabolism of slices of lactating mammary gland has been studied *in vitro*.

2 Rat mammary tissue shows an appreciable and maintained oxygen uptake in absence of substrate, but this is markedly increased in presence of glucose.

3 Mammary tissue from rats in full lactation gives a $-Q_{O_2}$ (approx $9 \mu\text{l}/\text{mg}$ final dry wt/hr) of the

same order as liver but less than that of very active tissues like retina and kidney. The order of activity of tissue from various species is inversely correlated with body size, being as follows: mouse, rat, guinea pig, rabbit, goat, cow.

4 In the presence of glucose the respiratory quotient (RQ) of tissue from the mouse, rat, guinea pig and rabbit is well above unity, but that of tissue from ruminants (goat, cow) is less than unity. The results therefore do not support the theory that the lower fatty acids of ruminant milk fat are synthesized from carbohydrate in the udder. The low RQ obtained in the absence of substrate in the rat suggests the oxidation of stored fat under these conditions.

5 Of a number of carbohydrates tested, including galactose, fructose and lactose, only mannose and glucose were oxidized by lactating rat mammary gland slices.

6 The aerobic acid production of rat mammary gland slices in presence of glucose is not high, but under anaerobic conditions is quite considerable. Despite the possibility that glycolysis may not account for all the acid produced, it may be concluded that this tissue exhibits a marked Pasteur effect.

7 Slices of mammary gland from rats in which lactation has been depressed by adrenalectomy on the fourth day of lactation or by partial manition show a lowered Q_{O_2} and RQ . The damaged cellular function is accompanied by a decrease in the Pasteur effect as evidenced by a significant increase in the apparent aerobic glycolysis.

We are indebted to Dr A. T. Cowie for performing adrenal ectomies and mammary gland biopsies, to Prof F. Dickens, F.R.S., for advice on manometric technique and to Prof M. Stacey for specimens of D-arabinose, glucose 1 phosphate and L-sorbose. We would also like to express our thanks to Mr J. F. Kayser of Messrs Gillette Industries Ltd for a generous gift of special razor blades, and to Mr A. S. Foot, Prof. H. G. Sanders and members of the staff of the Ministry of Food for making it possible for us to obtain lactating bovine udder tissue. Part of this work was done during the tenure by one of us (T. H. F.) of a research grant from the Agricultural Research Council.

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Observations on the Mono-amine Oxidase Activity of Placenta and Uterus

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(Received 4 February 1949)

It is known that under aerobic conditions the kidney can oxidize mono amines such as tyramine or adrenaline by means of a mono amine oxidase (Blaschko, Richter & Schlossmann, 1937), and it has also been shown that under anaerobic conditions it can decarboxylate tyrosine, dihydroxyphenylalanine and histidine (Werle, 1936, Werle & Hermann, 1937, Holtz, 1937, Holtz, Heise & Spreyer, 1938). More recently a number of experimental findings have suggested the possibility of the excessive production or defective inactivation of pressor amines playing a part in the production of certain types of hypertension (Blaschko, 1942)

The ideas that have been put forward in this connexion have been summarized by Schales (1947), who, in a discussion of the relation of kidney enzymes to essential hypertension, mentions the view that since mono amine oxidase requires oxygen to inactivate amines 'the restriction of renal circulation in experimental hypertension might make it possible for some pressor amines to escape unoxidized from the kidney', and so play a part in producing peripheral vaso constriction. Duff, Hamilton & Magner (1939) claim to have produced arteriolar lesions similar to those of malignant hypertension by repeated intravenous injection of tyramine into rabbits. Raska (1943) has reported that the mono amine oxidase activity of kidney slices from dogs rendered hypertensive by the Goldblatt technique is decreased. His results do not suggest that this decrease is specific since the rates of residual respiration (i.e. in the absence of added substrate) and of oxidation of DL-alanine, L-dihydroxyphenylalanine (DOPA) and histamine were also diminished. In support of this, Gerbi, Rubinstein & Goldblatt (1940) had earlier found a diminution of the residual oxygen consumption in ischaemic rabbit kidney. Mason, Robinson & Blalock (1940), on the other hand, compared the Q_{O_2} of renal cortical tissue from normal and hypertensive dogs in the presence of glucose and found no significant

difference, although they state that preliminary observations showed a striking difference in the rates of residual respiration and of ammonia formation.

Rather more indirect evidence suggesting the possibility of faulty inactivation of the pressor amine formed by decarboxylation of L-DOPA in hypertensive animals has been provided by experiments showing that perfusion of the ischaemic mammalian kidney with DOPA gives rise to the production of a pressor substance having the properties of hydroxytyramine (Bing, 1941). Moreover, the injection of DOPA into cats with experimental renal hypertension causes a marked rise in blood pressure, whereas in normal cats no effect was observed (Bing & Zucker, 1941, Oster & Sorkin, 1942).

In view of the presence in the kidney of this amine inactivating enzyme, and the suggestions which have been put forward regarding its possible relation to experimental renal hypertension, it was decided to study the possibility of some similar enzymic mechanism being a contributory factor in the development of the hypertension which is a marked and early feature of certain of the toxæmias of pregnancy. In this connexion the finding by Bhagvat, Blaschko & Richter (1939) of a high mono amine oxidase activity in normal uterus is of interest.

For these reasons a study was undertaken to determine whether an active mono amine oxidase is present in placenta, since this organ is thought by many workers to play an important part in the pathogenesis of the toxæmia, and also to investigate in greater detail the activity and properties of this enzyme in uterine tissue. Before this work was completed a brief report (Luschinsky & Singher, 1948) appeared on the identification of mono amine oxidase in human placenta. The present work confirms this observation and describes certain other properties of the enzyme in connexion with the physiology of placental tissue.

METHODS

Estimation of enzymic activity

Oxygen consumption was determined manometrically using the Warburg technique. In all cases, except where stated, the tissue was finely minced with scissors and then dispersed in 0.067M phosphate buffer, pH 7.3, in a high speed homogenizer of the type described by Folley & Watson (1948), so that 2 ml of the final homogenate corresponded to 200 mg fresh tissue, where the amount of available tissue was not sufficient to provide a large enough volume for homogenization at this concentration, it was dispersed in the smallest possible volume.

Tyramine hydrochloride (L Light and Co Ltd, recrystallized from water) was used as substrate in 0.01M solution except where otherwise stated.

O₂ uptake, using 2 ml of the tissue homogenate, was measured at 38°, and in a gas phase of O₂, over a period of 1 hr, the results being expressed as $\mu\text{l O}_2/\text{g fresh tissue/hr}$. Controls for non enzymic oxidation of the substrate, and for the O₂ consumption of the enzyme preparation in the absence of added substrate, were included in each experiment.

In experiments where it was desired to trap the aldehyde formed during the initial oxidation of the amine in order to prevent further oxidation occurring, semicarbazide hydrochloride was added to the reaction mixture to give a final concentration of 0.02M.

Ammonia was estimated using the Markham (1942) apparatus.

Materials

Rat, rabbit and guinea pig placenta The pregnant uterus was removed immediately after killing the animal by exsanguination. The placentas were then dissected free from the other products of conception, washed with 0.9% NaCl, blotted dry with filter paper, weighed and homogenized.

Human placentas were brought to the laboratory either immediately after delivery or after having been placed immediately in a refrigerator in which they remained for periods up to 10 hr. In most cases they were expelled *per vaginam*, although a few were obtained after Caesarian section. They were prepared by one of two methods.

(1) A small piece, approximately 2 cm square and extending through the entire thickness of the organ, was excised, mopped free from surface blood, washed with distilled water, weighed and homogenized.

(2) The membranes and larger blood vessels were dissected off, and the remaining tissue squeezed free from as much of the contained blood as possible, after which it was washed with glass-distilled water, mopped with filter paper, weighed, and passed through a Latapie mincer. The mince was then well mixed, and a weighed sample taken for homogenization.

Rat and rabbit uterus Immediately after killing the animals by exsanguination the uterus was removed, and, in the case of non pregnant animals, weighed and homogenized. When taken from pregnant animals the whole uterus was removed intact and weighed, if sufficiently developed, the products of conception were then dissected away, and the uterus was mopped free from blood and amniotic fluid with filter paper, washed rapidly in 0.9% NaCl, weighed and homogenized.

RESULTS

Mono amine oxidase activity of uterus

The presence of an active amine oxidase in dog and sheep uterus was demonstrated by Bhagvat *et al* (1939). We have extended this finding to the non pregnant uterus of the rat and rabbit. The results of six typical experiments are shown in Table 1.

Table 1 *Mono-amine oxidase activity of non-pregnant rat and rabbit uterus*

(Activity expressed in $\mu\text{l O}_2/\text{g fresh tissue/hr}$)

Rat	Rabbit
206	505
451	489
222	692
440	500
580	628
322	621

As the object of this work was a study of the enzyme in the pregnant animal, estimations were next made on the rat uterus at various stages of pregnancy. Thirty pregnant rats were used, and

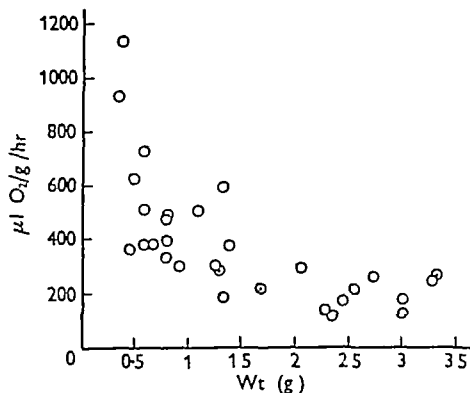


Fig 1 Relation between mono amine oxidase activity of pregnant rat uterus (expressed as $\mu\text{l O}_2/\text{g fresh tissue/hr}$) and the weight of the uterus after removal of the products of conception.

a scatter diagram relating the enzyme activities (expressed per g fresh tissue) to the weight of the uterus after removal of the products of conception is shown in Fig 1. It is realized that the distribution of such weights affords only an approximate indication of the duration of pregnancy, but owing to the wide variations in the number of fetuses contained in different animals the total weight of uterus, together with products of conception, provides an even less accurate indication.

From the results given in Fig 1 it will be seen that very high values for enzymic activity (up to $1130 \mu\text{l O}_2/\text{g/hr}$) are obtained early in pregnancy,

these values tending to fall as pregnancy advances to a low level of 100–300 $\mu\text{l O}_2/\text{g/hr}$ shortly before delivery

That the relatively high values obtained in the small uterus of early pregnancy are not due merely to the homogenate being necessarily more dilute, with the possibility therefore of an accompanying dilution of some inhibitory factor, is shown by the fact that equally dilute homogenates of non pregnant uterus do not show these high values. Further, in order to control this point in the pregnant uterus, a direct comparison was made of a uterus using homogenates of three different dilutions, no significant difference between the activities of the three dilutions was observed.

From our present experimental findings it is not possible to interpret this fall in activity per unit weight as pregnancy advances, but it suggests that the enzyme may be localized predominantly in the endometrium, and that the relatively greater mass of myometrium present late in pregnancy may be responsible for the lower activity per unit weight of the whole uterus. With this possibility in view, estimations were carried out on the separated endometrium and myometrium from two human uteruses.

The first was obtained from a normal pregnancy terminated by total hysterectomy at 17 weeks on account of a rapidly growing carcinoma of the breast. The decidua (endometrium in pregnancy), which was thick and plentiful, was separated with a scalpel from the myometrium, and homogenates were made of the two portions of the tissue, the activities of which were measured simultaneously in the usual way, the decidua showed an activity of 663 $\mu\text{l O}_2/\text{g/hr}$, and the myometrium 97 $\mu\text{l O}_2/\text{g/hr}$. The second uterus, which was non pregnant, was removed on account of menorrhagia, and gave values of 432 $\mu\text{l O}_2/\text{g/hr}$ for the endometrium and 171 $\mu\text{l O}_2/\text{g/hr}$ for the myometrium.

Two further specimens of human decidua have been obtained by Caesarian section at full term, and have given values of 1125 and 1016 $\mu\text{l O}_2/\text{g/hr}$.

Mono-amine oxidase activity of placenta

In view of the level of enzymic activity shown by human uterine tissue, human placenta was next investigated. Using small random samples excised from the placenta (method 1, see p 126), it was found that this tissue was also highly active, values ranging from 338 to 995 $\mu\text{l O}_2/\text{g/hr}$ being obtained in ten different placentas.

As mentioned above, Raska (1943) has reported a lowering of the mono amine oxidase level in kidneys from animals rendered hypertensive by the application of the Goldblatt clamp, and since normal human placenta, as well as kidney, appears to be a rich source of the enzyme, it was decided to attempt a comparison of the activity of this enzyme

in normal placentas and in those from cases of toxæmia of pregnancy showing hypertension.

The wide variations, however, in the results quoted above obtained on isolated samples of placental tissue led us, when embarking on this comparison, to use homogenates prepared from a portion of a well-mixed mince of the whole placenta, carried out as described above under method 2. Even when using this method it was found that considerable variation exists between the activities of different placentas.

Table 2 *Mono-amine oxidase activities of normal human placentas*

$\mu\text{l O}_2/\text{g fresh tissue/hr}$	mmol $\text{O}_2/\text{whole placenta/hr}$
665	16.5
510	9.6
660	16.5
660	12.0
925	10.2
535	8.1
406	8.2
705	11.6
825	14.3
808	14.0
555	14.0
825	10.1

The results obtained with twelve different normal placentas at full term are shown in Table 2, where the activities are given both in terms of $\mu\text{l O}_2/\text{g fresh tissue/hr}$ and also as mmol $\text{O}_2/\text{whole placenta/hr}$. From these figures, and from the published work on other human tissues (Birkhauser, 1940, Zeller, Stern & Wenk, 1940, Blaschko, 1942, Epps, 1945), it seems that normal human placenta at term must be classed as a tissue relatively rich in mono amine oxidase.

An active enzyme was also found in rat, rabbit and guinea pig placenta. In the course of these experiments with animal placentas variations in the level of activity of the mono amine oxidase were noted in placentas of different ages in different animals. A series of estimations was therefore carried out in rats at various stages of placental development. The results of these experiments are shown in Fig 2, in which enzymic activities of the placental homogenates from different animals (expressed as $\mu\text{l O}_2/\text{g fresh tissue/hr}$) are plotted against the mean placental weight for each animal.

From the results obtained it will be seen that the level of enzymic activity, expressed per g of placental tissue, appears to increase as the placenta ages. A linear regression line has been fitted, and has a slope of 55 $\mu\text{l O}_2/\text{g/hr}$ for each 0.1 g increase in mean placental weight. The correlation coefficient for the values shown in Fig 2 is 0.76 ± 0.21 . This correlation is in contrast to the results obtained with the whole uterus mentioned above.

If, as has been postulated, any relationship exists between mono amine oxidase activity in the kidney and experimental renal hypertension, the dependence of enzymic activity on O_2 tension and the relation of this to restriction of the renal circulation becomes important. Further, in view of the postulated association of placental ischaemia with pregnancy toxæmia, this relationship may also be of importance in connexion with the hypertension accompanying this condition. Kohn (1937) has shown that the mono amine oxidase of pig liver is unusually sensitive to changes in O_2 tension, the rate of oxidation of tyramine being increased by 200–250% by increasing the concentration of O_2 in the gas phase from 20 to 100%. By contrast he showed that a milk xanthine oxidase preparation was almost saturated at 20% O_2 .

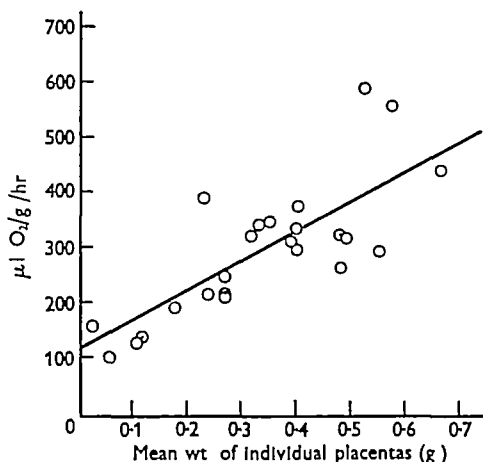


Fig 2 Relation between mono amine oxidase activity of rat placenta (expressed as $\mu l O_2/g$ fresh tissue/hr) and mean weight of the individual placentas from any one animal.

Using homogenates of human placenta we have compared the rates of oxidation of tyramine and of succinate at different oxygen tensions. A final concentration of 0.05M sodium succinate was used as substrate in the succinic oxidase determinations.

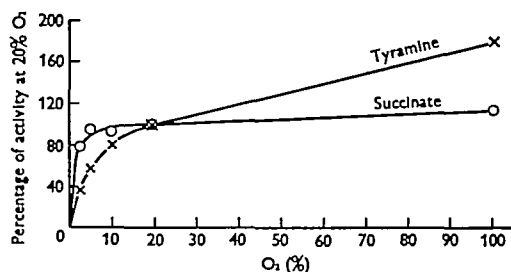


Fig 3 Effect of O_2 tension on rates of oxidation of tyramine and succinate by human placental homogenates (expressed as percentages of activity at 20% O_2)

The results of these experiments are shown in Fig 3, in which the enzymic activities are expressed as percentages of the activity at 20% O_2 . It will be seen that whereas the rate of oxidation of succinate

is only slightly affected by increasing the concentration of oxygen above about 25–5%, the rate of oxidation of tyramine increases strikingly as the oxygen tension is increased, the rate in the presence of 25% O_2 being less than half that in 100% O_2 . In addition to the enzyme in liver, the placental mono amine oxidase is therefore also highly sensitive to changes in oxygen tension, and any fall in the latter, such as might occur in ischaemic areas of placenta, might therefore be expected to cause a more pronounced fall in the activity of the mono amine oxidase than in that of certain other enzymes.

Specificity of the placental mono amine oxidase

The tyramine oxidase first described by Hare (1928) in liver was shown by Blaschko *et al* (1937) to have a wide range of specificity, oxidizing a number of aliphatic and aromatic mono amines. In the present work, in addition to tyramine, we have studied the oxidation of 2 phenylethylamine (L. Light and Co.), and tryptamine and DL-adrenaline (British Drug Houses Ltd.), by human placental extracts. These substrates were also used in final concentrations of 0.01M, the estimations of enzymic activity being carried out as with tyramine. In these experiments, however, semicarbazide was added to prevent the further oxidation of the aldehyde formed by deamination, together with 0.001M cyanide to inhibit the oxidation of adrenaline by the cytochrome oxidase system. KCN was also added to the centre cup to prevent loss of cyanide from the main chamber.

The results of these experiments are shown in Table 3. The relative rates of oxidation of tyramine, tryptamine and 2 phenylethylamine by human placenta are of the same order as those found by Blaschko *et al* (1937) for guinea-pig liver. The value quoted for adrenaline is somewhat higher, but although when using this substrate readings were taken every 10 min, wide variations in its rate of oxidation were found in different experiments.

Table 3 *Relative rates of oxidation of some aromatic amines by placental mono amine oxidase*

(Extra O_2 uptake due to tyramine/g/hr taken as 100. Reactions in the presence of 0.02M semicarbazide hydrochloride and 0.001M KCN)

Tyramine	100
Tryptamine	77
Adrenaline	128
2 Phenylethylamine	16

A number of experiments were carried out using 0.01M histamine dihydrochloride and cadavarine dihydrochloride as substrates, but in each case oxidation by placental homogenates under our conditions was barely measurable in the course of 1 hr.

Ammonia production by placental mono-amine oxidase

The rate of ammonia production has been estimated in a few experiments in order to correlate the extra oxygen uptake with deamination. At the

end of the measurement of oxygen uptake 1 ml of 25% trichloroacetic acid was added to each bottle. After standing 15 min the contents of the bottles were centrifuged, and the bottles and the precipitate washed twice with 1 ml of 5% trichloroacetic acid. The filtrates were then made up to 10 ml in volumetric flasks, and samples of each taken for ammonia estimation.

In each of these experiments the oxygen consumption in the presence of semicarbazide was also estimated, in order to determine the proportion of total oxygen uptake by the placental enzyme which was due to oxidation of the aldehyde formed. The results of these experiments are shown in Table 4.

Table 4 Ratio of atoms oxygen utilized/moles ammonia produced, and ratio of oxygen utilized in the presence and absence of semicarbazide, during oxidation of tyramine by the placental enzyme

O ₂ uptake (μ l O ₂ /g /hr)		NH ₃ produced (μ M/g /hr)	Atoms O/moles NH ₃		Ratio of O ₂ uptakes (a)/(b)
Without semicarbazide (a)	With semicarbazide (b)		Without semicarbazide	With semicarbazide	
672	521	43.9	1.37	1.06	1.29
601	506	48.6	1.10	0.92	1.19
680	595	52.4	1.16	1.01	1.16
890	735	55.7	1.42	1.17	1.21

In the absence of semicarbazide a mean O/NH₃ ratio of 1.26 was obtained, when, however, the figures for the oxygen uptake in the presence of semicarbazide are used, the ratios in these four experiments become 1.06, 0.92, 1.01 and 1.17 respectively.

DISCUSSION

The findings reported in this paper establish the presence of an active mono amine oxidase in placental extracts, confirming the recent observations of Luschinsky & Singher (1948). From a study of its action on different amines it has been shown that this enzyme, like the mono amine oxidase present in liver, kidney and elsewhere, can oxidize a number of aromatic mono amines, but is inactive against diamines such as histamine or cadaverine.

In human placenta at term, this enzyme is present in relatively large amounts, and in rat placenta its activity appears to increase as the placenta matures. From the experiments which we have made it is impossible to do more than speculate on the possible significance of this enzyme in placenta, but the fact that in rat placenta its activity appears to increase as the placenta matures suggests that it may exercise some function of increasing significance as pregnancy advances, and the foetus or foetuses increase in size and differentiation. In this connexion it should be noted that Epps (1945) has shown that the kidney cortex and medulla of the newborn child are deficient in mono amine oxidase, the activity

gradually increasing until the child is about 3 months old, when maximum activity appears to be reached. In a study of the DOPA decarboxylase activity of various human and animal tissues Page (1945), using a biological method for estimating the hydroxy tyramine formed, was unable to detect the presence of this enzyme in human placenta. As far as we are aware, however, the decarboxylation of other amino acids by placental or foetal tissue has not so far been studied.

The demonstration by Kohn (1937) that the activity of the mono amine oxidase of liver is determined by the oxygen tension has been shown to apply also to the enzyme in placenta. In the absence

of any exact information as to the oxygen tensions to which the enzyme would normally be exposed under *in vivo* conditions, it is clearly impossible to interpret this property of amine oxidase in any physiological sense. It does suggest, however, that any lowering of the local oxygen tension, such as might be expected to occur in the placenta, more particularly towards the end of pregnancy, might result in an inhibition of the activity of this enzyme to a greater extent than that of the succinic oxidase system, and possibly also of other systems involving the cytochrome cytochrome oxidase mechanism.

The high values of the mono amine oxidase activity of the rat uterus early in pregnancy together with the apparent fall in these values, when expressed on a weight basis as pregnancy advances, suggests that the enzyme may be chiefly localized in the endometrium, and this supposition has been confirmed by examination of the separate endometrium and myometrium from human uterus. The low value found in the myometrium would indicate that this muscle is similar to the other muscular organs examined by Bhagvat *et al* (1939), the high value of whole uterus being due mainly to an endometrial enzyme.

Experiments are now in progress in the hope of obtaining information concerning the physiological significance of this enzyme in placenta, and to ascertain whether any abnormalities of its function are detectable in the toxemias of pregnancy.

SUMMARY

1 The presence of an active mono amine oxidase in uterus has been confirmed. Evidence is presented indicating that in the human uterus, and possibly also in the rat uterus, it is mainly localized in the endometrium.

2 Human, rat, rabbit and guinea pig placentas have also been found to be potent sources of the enzyme.

3 The level of enzymic activity in rat placenta (expressed per g of tissue) appears to increase as the placenta matures *in utero*.

4 The activity of the placental enzyme, like that of the enzyme in liver, is proportional to the oxygen tension of the medium in which it acts, a fall of oxygen tension causing a marked fall in rate of enzyme action.

We would like to acknowledge the help given to us in the collection of human material by Mr J B Blackley, F R C S, F R C O G, and the staff of the Department of Obstetrics and Gynaecology, Guy's Hospital Medical School. Our thanks are also due to Mr R F Adams and Miss E Smalley for technical assistance.

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Effect of Sulphydryl-combining Compounds on the Activity of the Succinic Oxidase System

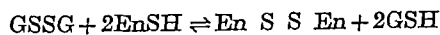
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(Received 22 November 1948)

Thunberg (1916) was the first to suggest that the tissue catalyst responsible for the oxidation of succinate contained a sulphydryl group, but his evidence, viz that colloidal sulphur inhibited the enzyme, was not very convincing. There was, in fact, better evidence available to Thunberg, since Battelli & Stern (1910) had already shown that arsenite inhibited this enzyme, and Ehrlich (1909) had suggested that the toxic action of arsenicals on tissues might be due to their combination with sulphydryl groups. This suggestion of Ehrlich has recently received convincing experimental support by the work of Peters and his colleagues (Peters, Stocken & Thompson, 1945).

Hopkins and his co workers (Hopkins & Morgan, 1938, Hopkins, Morgan & Lutwak Mann, 1938) were

the first to demonstrate the existence and the importance of SH groups in succinic dehydrogenase. These workers showed that succinic dehydrogenase was completely inhibited by incubation with a high concentration of oxidized glutathione (GSSG), and that the activity was completely restored by incubating the inhibited enzyme with reduced glutathione (GSH) under anaerobic conditions. They suggested that GSSG acted by oxidizing the SH groups of succinic dehydrogenase to disulphide (S S) groups and that this reaction was reversible



Since the publication of the papers of Hopkins and his co workers, many studies of the action of other SH combining compounds on succinic oxidase

have appeared (e.g. Morgan & Friedmann, 1938, Potter & Dubois, 1943, Barron & Singer, 1945, Barron & Kalnitsky, 1947, Mackworth, 1948). Five different types of compounds have been used, viz alkylating agents, oxidizing agents, arsenicals, mercaptide forming agents and maleic acid.

Despite these many studies of the action of SH agents, an examination of the literature revealed that certain points required further investigation. The most important of these was the reversibility of the inhibition, concerning which the literature presents a confusing picture. Hopkins *et al* (1938) showed that treatment with GSH completely reactivated the succinic oxidase system in washed minced muscle after it had been inactivated by GSSG. Potter & Dubois (1943), on the other hand, using a tissue homogenate as the source of the enzyme, were unable, by means of GSH, to reverse the inhibition caused by various oxidizing agents. They explained the disagreement between their findings and those of Hopkins *et al* by pointing out an important difference in the experimental procedures. Hopkins *et al* treated the inactivated enzyme with GSH under anaerobic conditions, and, after this treatment, removed the excess GSH by filtering and washing the muscle pulp. Since this procedure is not possible when dispersions are used as the source of the enzyme, Potter & Dubois did not remove the GSH before measuring the succinic oxidase activity. GSH is fairly rapidly oxidized when added to such tissue preparations in the presence of air (especially if excess cytochrome *c* is added), and so cannot be an effective reactivating agent. In fact, Potter & Dubois (1943) and Ames & Elvehjem (1944) found that GSH and cysteine actually inhibited the succinic oxidase activities of tissue preparations. The presence of the thiol in the reaction mixture during the measurement of the activity of the enzyme introduces a further difficulty, viz that the absorption of oxygen causes an error in the determination of the succinic oxidase activity. In opposition to the findings of Potter & Dubois, Barron & Singer (1945) claim that the succinic oxidase activity of a muscle dispersion, previously inhibited to the extent of 80% by treatment with benzoquinone or *p* carboxyphenylarsine oxide, was completely restored by treatment with GSH (50 times the concentration of the inhibitor). These authors state that the 'reactivation produced by glutathione was instantaneous and in no way connected with its oxidation by metal catalysts in the enzyme, since the enzyme suspension showed no appreciable oxygen uptake on addition of glutathione without succinate during the course of the experiment (20 min)'. Barron, Miller, Bartlett, Meyer & Singer (1947) later showed that the dithiol 2,3-dimercaptopropanol (BAL) was more effective than GSH in reversing the inhibition by arsenicals.

A further point requiring study is the component of the succinic oxidase system which is attacked by the inhibitor. Some authors, although using the rate of oxygen uptake in the presence of succinate as the measure of the enzyme activity, speak of the enzyme concerned in this oxidation as succinic dehydrogenase. Actually, an enzyme complex, of which succinic dehydrogenase is only one component, is necessary to catalyse the aerobic oxidation of succinate.

An additional reason for these experiments was to study the action of SH combining compounds on the succinic oxidase system in Keilin & Hartree's heart muscle preparation, which, although it is the most active preparation of this enzyme system, has not been used by previous investigators studying these inhibitors.

Three types of inhibitors were used, viz (1) oxidizing agents *o* iodosobenzoate and GSSG, (2) *p* aminophenylarsenoxide, (3) *p* chloromercuribenzoate. Alkylating agents were not studied, since this inhibition is not easily reversible. A preliminary account of some of these findings has already appeared (Slater, 1948).

METHODS

Enzyme preparation. Keilin & Hartree's (1947) heart-muscle preparation was used. The methods of preparing the enzymes and determining the activities have already been described (Slater, 1948*a, b*).

Glutathione was prepared by the methods of Hopkins (1929) and Pirie (1930). Aqueous solutions were neutralized immediately before use. GSSG was prepared by shaking a neutralized solution of GSH in a manometric flask until no more O_2 was absorbed.

BAL was kindly provided by Prof R. H. S. Thompson. Aqueous solutions were prepared immediately before use.

o Iodosobenzoic acid was prepared by the oxidation of *o*-iodobenzoic acid with fuming HNO_3 , according to the method of Meyer & Wachter (1892). It was 90% pure according to the amount of I_2 liberated from acidified KI.

p Chloromercuribenzoate was kindly supplied by Dr S. V. Perry.

Procedure. The general procedure adopted was to treat a concentrated enzyme preparation with the inhibitor for a definite length of time and then rapidly to dilute about 50 fold before measuring the enzyme activity. Except in the case of *p* chloromercuribenzoate, this rapid dilution practically stopped the action of the inhibitor on the enzyme. Unless otherwise stated, 1 ml of Keilin & Hartree's (1947) heart-muscle preparation was treated with the inhibitor solution, after standing for the required time, the solution was diluted to 5 ml. with 0.18M phosphate buffer, pH 7.3, and 0.2 ml samples were pipetted into the manometer flasks and immediately diluted to 3.1 ml. with phosphate buffer and the other solutions necessary for the measurement of the enzyme activity, which was carried out as soon as possible.

This method is valid only if dilution of the enzyme does not cause any reversal of the inhibitions. This was found to be the case.

EXPERIMENTAL

A Effect of sulphhydryl combining agents on the succinic oxidase system

(1) *Rate of inhibition* The effect of the time of contact of the inhibitor with the enzyme at 37° on the degree of inhibition is shown for four inhibitors in Fig 1. The figures at zero time were obtained by adding the inhibitor and immediately diluting. The small inhibitions found with *o*-iodosobenzoate and *p* aminophenylarsenoxide at zero time probably occurred during this dilution or after the dilution.

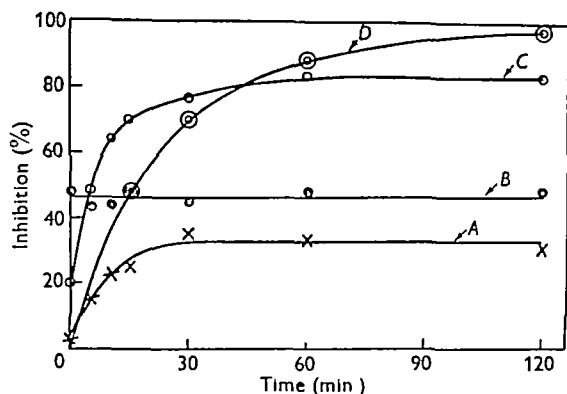


Fig 1 Rate of inactivation, at 37°, of succinic oxidase system by 0.2 μ equiv *o*-iodosobenzoate per ml heart-muscle preparation (curve A), 0.5 μ equiv *p* chloromercuribenzoate per ml heart-muscle preparation (curve B), 0.2 μ equiv *p* aminophenylarsenoxide per ml heart-muscle preparation (curve C), 20 μ equiv GSSG per ml heart-muscle preparation (curve D)

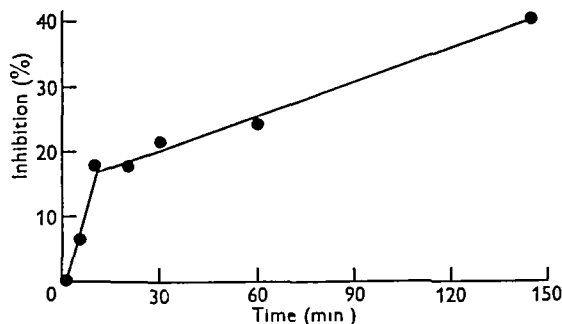


Fig 2 Rate of inactivation, at 16°, of the succinic oxidase system by 2 μ equiv *o*-iodosobenzoate per ml heart-muscle preparation.

Inhibition by *p* aminophenylarsenoxide and by *o*-iodosobenzoate, and particularly by GSSG (even in large excess) was a slow process, maximum inhibition was found with the arsenical and *o*-iodosobenzoate after 1 hr. The reaction between the enzyme and *p* chloromercuribenzoate was much more rapid. It cannot be stated from Fig 1 whether this in-

hibition occurred practically instantaneously before or after dilution. It can, however, be definitely concluded that, under comparable conditions, the rate of inhibition of the enzyme by *p* chloromercuribenzoate is much greater than that by the other inhibitors tried. The succinic dehydrogenase, which, as is shown below, is less susceptible to the mercurial than is the complete succinic oxidase system, was just as rapidly inactivated by the mercurial.

In Fig 2, the rate of inhibition of the enzyme by *o*-iodosobenzoate at 16° is shown. It appears from the shape of the curve that the inhibition at this temperature was due to two reactions, an initial fairly rapid reaction which was complete after 10 min, followed by a slower reaction, which was not complete after 2 hr.

(2) *Effect of amount of inhibitor* The effect of different amounts of *o*-iodosobenzoate, *p* aminophenylarsenoxide and *p* chloromercuribenzoate on the succinic oxidase activity of the same heart muscle preparation is shown in Fig 3. Sufficient time was allowed for complete reaction between the

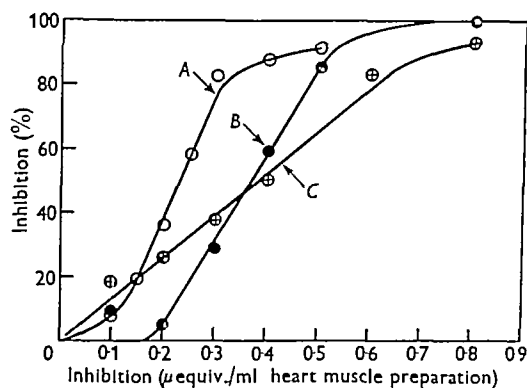


Fig 3 Effect of different amounts of *p* aminophenylarsenoxide (curve A), *o*-iodosobenzoate (curve B), and *p* chloromercuribenzoate (curve C) on the succinic oxidase system. *p*-Amino phenylarsenoxide and *o*-iodosobenzoate reaction carried out at 37° for 60 min., *p* chloromercuribenzoate 37° for 15 min.

inhibitor and the enzyme. A considerable amount of the arsenical or *o*-iodosobenzoate was required before appreciable inhibition occurred, the inhibition increased markedly with further small increases in the amount of inhibitor until nearly complete inactivation was obtained. The unusual shape of the curves is probably due to the presence, in the heart muscle preparation, of substances (possibly denatured proteins, which are known to contain many reactive SH groups) which combine preferentially with these two inhibitors and thus protect the enzyme from inactivation by small concentrations of the inhibitor. The heart muscle preparation often became less susceptible to SH combining reagents (particularly to *o*-iodosobenzoate) after standing for a few days,

probably due to an increase in the amount of denatured proteins

These substances in the heart muscle preparation which protected the enzyme from small amounts of arsenical and oxidizing agent were quite ineffective in protecting against the same amount of the mercurial. On the other hand, the enzyme was more susceptible to relatively larger amounts of the arsenical and *o* iodosobenzoate than to the corresponding amount of mercurial. These findings indicate that the mercurial was much less selective in its action than the other two compounds. The arsenical was the most selective agent of the three.

(3) *Component of the succinic oxidase system inhibited by the sulphhydryl reagents*. In Fig 4 the effect of different amounts of *p* aminophenylarsenoxide on the activity of both the complete succinic oxidase system and on the succinic dehydrogenase

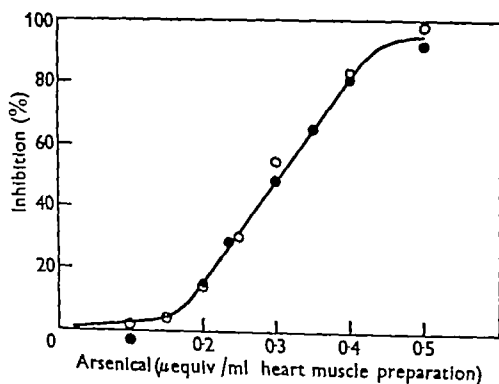


Fig 4 Effect of different amounts of *p* aminophenylarsenoxide on the succinic oxidase system ○, and on succinic dehydrogenase ●. Reaction carried out at 37° for 60 min.

portion is shown. The fact that the two curves coincide means that the component of the succinic oxidase system which is limiting the activity of the system after treatment with the arsenical is the succinic dehydrogenase, and that the primary action of the arsenical is on the dehydrogenase.

The oxidizing agents, *o* iodosobenzoate and GSSG, on the other hand, have a greater effect on the complete system than on the succinic dehydrogenase (Figs 5 and 6). Cupric chloride, which may act primarily as an oxidizing agent, also has a much stronger action on the complete system than on the dehydrogenase. Hellerman, Perkins & Clark (1933) and Mapson (1946) have shown that Cu^{++} does not rapidly inactivate urease, whereas Cu^+ is strongly inhibitory. Hopkins (1929) found that GSH reacted with Cu^{++} by first reducing it to Cu^+ , which then combined with unreacted GSH. It is possible that the same type of reaction occurs with the succinic oxidase system, the first action of the Cu^{++} may be an oxidative reaction on some component of the

system, leading to inactivation of the succinic oxidase system, and this is followed by combination of the Cu^+ formed with the SH groups of the succinic dehydrogenase.

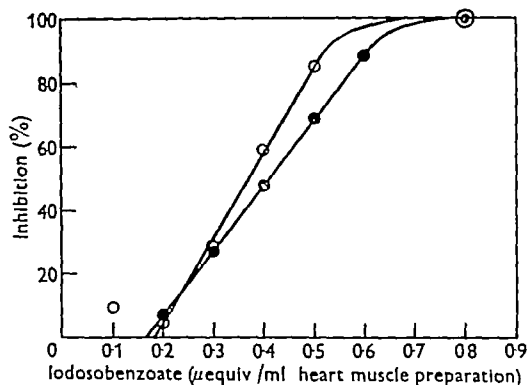


Fig 5 Effect of different amounts of iodosobenzoate on the activity of succinic oxidase ○, and of the succinic dehydrogenase ●. Reaction carried out at 37° for 60 min.

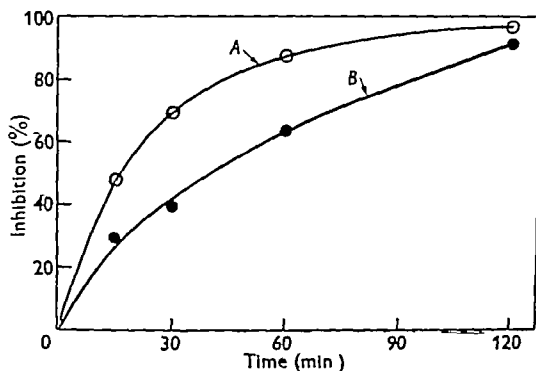


Fig 6 Rate of inactivation of succinic oxidase (○, curve A) and succinic dehydrogenase (●, curve B) by GSSG (20 μequiv/ml heart-muscle preparation). Temp 37°.

Since Hellerman *et al* (1933) found that Cu^{++} catalysed the aerobic destruction of urease, it was of interest to determine whether it had the same effect on the succinic oxidase system. The figures in Table 1 show that this is not the case, Cu^{++} had the same inhibitory effect whether it was tested in the presence or in the absence of air.

The effect of different amounts of *p* chloromercuribenzoate on the succinic oxidase and succinic dehydrogenase activities is shown in Fig 7. The Thunberg method of measuring succinic dehydrogenase activity was employed, since potassium cyanide, which is used in the manometric method to inhibit cytochrome oxidase, reverses the inhibition of the dehydrogenase (see p 137). The mercurial, like the oxidizing agents but unlike the arsenical, inhibits the complete succinic oxidase system more than the dehydrogenase.

Table 1 *Effect of cupric ions on the succinic oxidase system and on succinic dehydrogenase*

(Heart muscle preparation (1 ml.) + 0.4 ml CuCl_2 were either shaken in a manometer flask in the presence of air (aerobic) or kept *in vacuo* (CuCl_2 added after evacuation) in a Thunberg tube (anaerobic) for 15 min at 37° . Concentration of Cu^{++} $7.2 \times 10^{-2} \text{M}$. 3.6 ml 0.18M phosphate buffer were then added and 0.2 ml samples taken for measurement of enzyme activities. Control without Cu^{++} treated in same way.)

	Succinic oxidase activity ($\mu\text{l O}_2/10 \text{ min}$)		Succinic dehydrogenase activity ($\mu\text{l O}_2/10 \text{ min}$)	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Control	112	110	41	40
In presence of Cu^{++}	46	44	29	27
Inhibition (%)	59	60	29	34

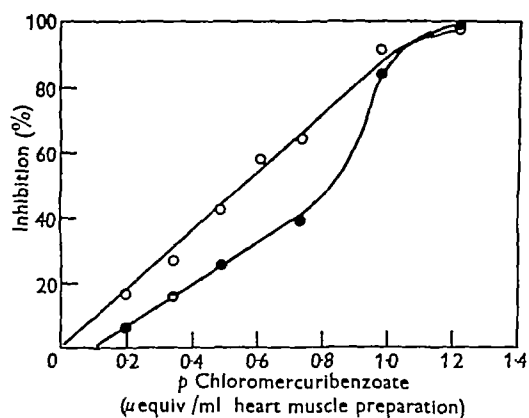


Fig 7 Effect of different amounts of *p* chloromercuribenzoate on the activity of succinic oxidase O, and of the succinic dehydrogenase ●. Reaction carried out at 37° for 15 min.

The effect of the arsenical, the mercurial and *o* iodosobenzoate on the cytochrome oxidase activity is shown in Table 2. Only the mercurial had an appreciable effect on the cytochrome oxidase. The insensitivity of cytochrome oxidase to arsenicals and oxidizing agents is in agreement with the findings of Hopkins *et al.* (1938) with GSSG and Barron & Singer (1945) with arsenicals.

Table 2 *Effect of sulphhydryl combining agents on the cytochrome oxidase of heart muscle preparation*

(Heart-muscle preparation treated with inhibitors for 1 hr at 37° . General procedure see Methods. Note that the cytochrome oxidase activity measured was the true activity at infinite cytochrome *c* concentration, measured by the method of Slater (1949b).)

Inhibitor	Inhibition (%)
<i>p</i> Chloromercuribenzoate (1.25 μequiv)	29
<i>o</i> Iodosobenzoate (1.0 μequiv)	5
<i>p</i> Aminophenylarsenoxide (0.5 μequiv)	5

B Reactivation

The inhibition produced by treatment with *p* aminophenylarsenoxide was not reversed when the concentration of the inhibitor was reduced by diluting the reaction mixture (Table 3), nor even by precipitating the enzyme with acid and washing away the unreacted inhibitor. Similarly, the inhibition by *o* iodosobenzoate was not reversed by the latter treatment (Table 4). After treatment with *p* chloromercuribenzoate also, the enzyme was not reactivated by dilution.

Table 3 *Effect of dilution of heart muscle preparation, after treatment with p-aminophenylarsenoxide, on the degree of inhibition*

(Inhibited 1 ml heart-muscle preparation + 0.5 ml 0.0004N *p* aminophenylarsenoxide kept at 37° for 1 hr, 3.5 ml 0.18M phosphate buffer added and different volumes pipetted into manometer flasks for measurement of succinic oxidase activity in the usual manner. Control 1 ml heart-muscle preparation + 0.5 ml water treated similarly.)

Final dilution of heart-muscle preparation in manometer flasks	O_2 uptake ($\mu\text{l}/10 \text{ min}$)		Inhibition (%)
	Control	Inhibited	
1/82	61	35	43
1/41	118	69	42
1/24	187	112	40
1/16	231	141	39

Table 4 *Inhibition of succinic oxidase by o-iodosobenzoate, attempt to reverse inhibition by washing enzyme with water*

(A, 1 ml heart-muscle preparation treated with *o* iodosobenzoate (total volume = 1.1 ml) for 30 min at 16° , 3.9 ml 0.15M phosphate buffer added. B, 1 ml heart-muscle preparation treated with *o* iodosobenzoate as A. Cooled to 0° and 4 ml 0.01N acetic acid added, centrifuged, supernatant fluid removed, residue stirred with 2 ml water, centrifuged and residue suspended in 5 ml 0.15M phosphate buffer. 0.2 ml samples taken for measurement of enzyme activity. Control tests showed that untreated enzyme could be precipitated with acid, washed and resuspended in phosphate buffer in this manner without loss of activity.)

<i>o</i> Iodosobenzoate (μequiv/ml heart-muscle preparation)	Inhibition (%)	
	A	B
2	71	78
4	85	83

The enzyme can, however, be reactivated by treatment with SH compounds. It was found, in agreement with Potter & Dubois (1943) and Ames & Elvehjem (1944) and in disagreement with Barron & Singer (1945), that complete reactivation of the succinic oxidase system could not be effected by adding the thiol to the enzyme preparation in the presence of air, since the thiol was oxidized by the

enzyme preparation and this oxidation was accompanied by inactivation of the succinic oxidase system. Ames & Elvehjem (1944) believed that this inhibition, caused by cysteine and GSH, was due to the action of cystine and GSSG formed by oxidation of the thiol compounds. It was found in the present study, however, that this could not be the complete explanation since the inhibition caused by GSH is much greater than that resulting from treatment with the equivalent concentration of GSSG. This observation was the starting point of the investigation of reducing agents on the succinic oxidase system which is described elsewhere (Slater, 1949c). It was found that GSH had no effect on the enzyme under completely anaerobic conditions, and that, even in the presence of air, the succinic dehydrogenase was much less susceptible than the complete succinic oxidase system to the thiol. Thus, in order to study the reversal of the inhibition, it is necessary to use Hopkins & Morgan's (1938) procedure of treating the enzyme with the thiol under anaerobic conditions. When using dispersions, it is difficult to remove the thiol from the enzyme quickly after the treatment before the measurement of the enzyme activity. This difficulty was overcome by treating a high concentration of enzyme with the thiol under anaerobic conditions and quickly diluting the suspension about 50 times before measuring the enzyme activity. The final very low concentration of the thiol was then without appreciable effect on the enzyme. In some experiments, such a high concentration of thiol was necessary to reactivate the enzyme that the thiol concentration, even after this 50 fold dilution, was appreciable and caused a small inactivation of the enzyme. In these experiments, the extent of the inactivation was determined in a separate control experiment and taken into account when calculating the degree of reactivation of the enzyme (see Fig 8).

In the presence of air, the relative effectiveness of different thiols in reversing the inhibition caused by SH combining reagents will depend, not only on the relative affinity of the thiol and the enzyme for the inhibitor, but also on the rate of oxidation of the thiol. The latter factor must be taken into account when considering the finding of Barron & Kalnitsky (1947) that several dithiols were more effective than BAL in reversing the inhibition of the succinic oxidase system caused by various heavy metals, since these authors worked under aerobic conditions and Barron, Miller & Kalnitsky (1947) had previously shown that these other dithiols were oxidized more slowly than BAL.

(1) *p* Aminophenylarsenoxide

(a) *Rate of reactivation* The heart muscle preparation, after treatment with 0.5 μ equiv of *p* aminophenylarsenoxide per ml of heart muscle

preparation for 30 min at 37°, was reactivated by treatment with BAL for various times before dilution. The results, which are given in Fig 8, show that the reactivation was quite rapid. It should be noted that the times of contact with BAL refer only to the time before dilution. It seems likely, from the

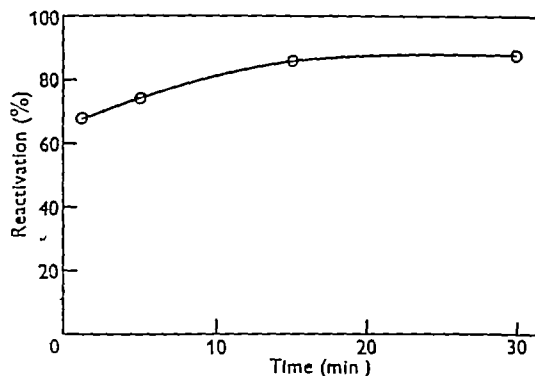


Fig 8 Rate of reactivation of succinic dehydrogenase, after inhibition by *p* aminophenylarsenoxide, by treatment with BAL under anaerobic conditions, equiv BAL/equiv *p* aminophenylarsenoxide = 3.76. Mixture A, 12 ml heart-muscle preparation + 6 ml water kept at 37° for 30 min., 2 ml A diluted with 4.4 ml 0.18M phosphate buffer, and succinic dehydrogenase activity of 0.2 ml of diluted solution determined, value *a*. Mixture B, 12 ml heart-muscle preparation + 6 ml 0.0005M *p* aminophenylarsenoxide kept at 37° for 30 min., 2 ml B diluted with 4.4 ml 0.18M phosphate buffer, and succinic dehydrogenase activity of 0.2 ml diluted solution determined, value *b*. Samples of A (2 ml) placed in Thunberg tubes, containing 0.25 ml 0.005M BAL in hollow stoppers. Evacuated, contents of Thunberg tubes mixed and reacted for various times before rapidly diluting with 4.4 ml 0.18M phosphate buffer, 0.2 ml of diluted solutions taken immediately for measurement of succinic dehydrogenase activity, value *c*. Samples of B (2 ml) treated in exactly the same manner, value *d*. Percentage inactivation = $100(a-b)/a = 88$, percentage reactivation = $100(ad/c - b)/(a-b)$.

shape of the curve, that some reaction took place either during or after dilution and the curve should not be taken to represent the true rate of reactivation. It can be seen, however, by comparison with Fig 1, obtained under the same conditions, that the rate of reactivation was considerably greater than the rate of inactivation.

(b) *Reactivation by different concentrations of BAL and GSH* The effect of different concentrations of BAL on the degree of reactivation is shown in Fig 9, which includes measurement both of succinic dehydrogenase and of the complete succinic oxidase system. The succinic dehydrogenase and the complete system were reactivated to about the same extent. Under the conditions of this experiment, the reactivation was not quite complete, amounting to

about 80% of full reactivation. This may be because, during the necessary manipulations, the enzyme-arsenical compound was allowed to stand for over 1 hr before the addition of the BAL. Barron & Singer (1945) found that complete reactivation of

the maximum reactivation obtained. When a similar experiment was carried out in an open test tube, BAL equivalent to 2.5 times the amount of arsenical reactivated to the extent of only 36%, even allowing for the inactivating effect of BAL itself. These findings may be compared with those of Barron, Miller, Bartlett, Meyer & Singer (1947), who found that BAL equivalent to 2.5 times the concentration of lewisite reactivated the succinic oxidase system by 83%, while double this amount completely reactivated the system. The amount of BAL required to reverse the inhibition of the succinic dehydrogenase by *p*-aminophenylarsenoxide is considerably less than that found by Stocken, Thompson & Whittaker (1947) to be necessary to reactivate the pyruvic oxidase system after the action of various therapeutic arsenicals.

GSH was much less effective than BAL in reversing the inhibition, thus, GSH equivalent to 1.5 times the concentration of arsenical produced no measurable reactivation, while 7.5 times the equivalent concentration caused 36% reactivation. This result is similar to that obtained by Barron, Miller, Bartlett, Meyer & Singer (1947), who found that GSH equal to 5 times the lewisite used reversed the inhibition by 23%.

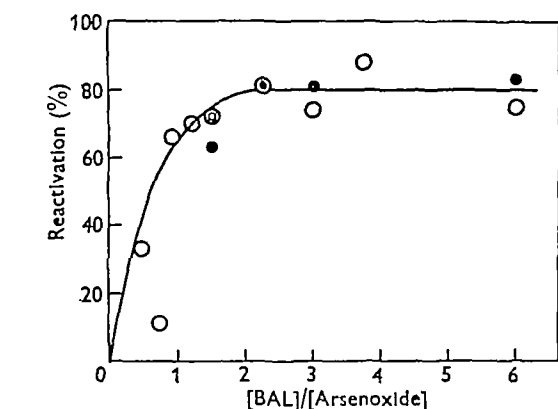


Fig 9 Reactivation of succinic dehydrogenase and succinic oxidase, after treatment with *p*-aminophenylarsenoxide (0.5 μ equiv/ml heart-muscle preparation), by treatment with various concentrations of BAL under anaerobic conditions. ●, succinic oxidase, ○, succinic dehydrogenase. Procedure as Fig 8, reactivation time, 30 min, inactivation, 85%.

the enzyme inhibited by *p*-chloromercuribenzoate occurred only when the thiol (GSH) was added soon after the mercurial and suggested that the enzyme whose SH groups are combined is readily denatured on standing. The most interesting feature of Fig 9 is the small amount of BAL required to produce the maximum reactivation, viz about twice the amount of BAL equivalent to the arsenical added, BAL exactly equivalent to the arsenical produced 80% of

(2) *o*-Iodosobenzoate

The experiments reported in Table 5 show that much greater amounts of thiol are required to reverse the inhibition caused by *o*-iodosobenzoate. BAL equivalent to 10 times the amount of oxidizing agent reactivated the succinic dehydrogenase by 61%, while BAL equivalent to 50 times the amount of iodosobenzoate brought about nearly complete reactivation. GSH was rather less effective than

Table 5 Reactivation of succinic dehydrogenase and of succinic oxidase after inhibition by *o*-iodosobenzoate

(Exp 1 5 ml heart-muscle preparation treated with 5 ml 0.001M *o*-iodosobenzoate for 30 min at 37°, 2 ml samples pipetted into Thunberg tubes containing BAL or GSH in hollow stoppers. Contents mixed after evacuation of tubes. After 30 min. at 37°, tube opened, contents quickly diluted to 5 ml. with phosphate buffer, 0.2 ml samples pipetted into manometer flasks and quickly diluted with buffer. Exp 2 Thunberg tubes contained 1 ml heart-muscle preparation and 0.3 ml 0.001M *o*-iodosobenzoate in main compartments and BAL or GSH in hollow stoppers. Evacuated, allowed to react for 30 min at 37°, then mixed and allowed to stand a further 30 min at 37°. Subsequent treatment as in Exp 1. Percentage reactivation calculated as in Fig 8.)

μequiv <i>o</i> -iodosobenzoate/ml heart-muscle preparation	Inhibition (%)		Reactivating agent	Ratio of BAL or GSH (equiv) <i>o</i> -iodosobenzoate (equiv)	Reactivation (%)	
	Succinic oxidase	Succinic dehydrogenase			Succinic oxidase	Succinic dehydrogenase
Exp 1						
2.0	—	57	BAL	1.5	—	12
			BAL	5	—	29
			GSH	1.5	—	0
Exp 2						
0.6	96	89	BAL	10.5	26	61
			BAL	50	54	92
			GSH	50	32	70

BAL The inhibition of the complete system was much less readily reversible than that of the succinic dehydrogenase

(3) Oxidized glutathione

Owing to the necessity of using very high concentrations of GSSG to inhibit the enzyme, it was not possible to use a large excess of thiol to reactivate the enzyme. In the experiment shown in full in Table 6, BAL, equivalent to twice the concentration

Table 6 *Reactivation of succinic dehydrogenase and succinic oxidase after treatment with oxidized glutathione*

(A, 1 ml heart-muscle preparation kept at 37° for 1 hr, diluted to 5 ml and succinic oxidase and succinic dehydrogenase determined on 0.2 ml. samples. B, 1 ml heart-muscle preparation + 0.2 ml. 0.05M GSSG (prepared by aerobic oxidation of neutralized 0.1M GSH) kept at 37° for 1 hr. Then as A. C, 1 ml. heart-muscle preparation in main tube and 0.2 ml 0.1M BAL in hollow stopper. Contents mixed after evacuation and kept at 37° for 30 min. Then as A. D, 1 ml. heart-muscle preparation in main tube and 0.2 ml. 0.1M BAL in hollow stopper. 0.2 ml 0.05M GSSG added to heart-muscle preparation, kept at 37° for 1 hr. Then evacuated, contents of hollow stopper mixed with those of main tube and kept at 37° for 30 min. Then as A.)

Exp	Succinic oxidase (μ l. O ₂ /10 min.)	Succinic dehydrogenase (μ l. O ₂ /20 min.)
A	101.7	62
B	46.5	41.1
C	78.9	55.2
D	16.6	53.6
Inhibition by GSSG (%)		
$\frac{A-B}{A} \times 100$	56	36
Reactivation by BAL (%)		
$\frac{AD/C-B}{A-B} \times 100$	-45	+91

of GSSG used to inhibit the enzyme, completely reactivated the succinic dehydrogenase, but did not reactivate the complete succinic oxidase system. In fact, the inhibition of the latter increased during the treatment with BAL. It is impossible to compare the relative ease of reversibility after treatment with o iodosobenzoate and GSSG, since the amount of the latter used was so much greater than that of the former that the concentration of BAL present in the experiment with GSSG was greater than in the experiment with o iodosobenzoate, although the relative concentrations of reactivating agent to inhibitor were much greater in the latter experiment. Further information on the reversibility of the enzyme system after treatment with GSSG could probably be obtained if the enzyme was sedimented by high speed centrifugation and washed free from GSSG before adding the reactivating agent.

Hopkins *et al* (1938) treated the washed muscle with GSSG under anaerobic conditions. It was found in the present study that the inhibitions of both the complete succinic oxidase system and of succinic dehydrogenase and the reactivations by BAL were the same, whether the enzyme was treated with the GSSG in the presence or in the absence of air.

Inhibition by GSSG was not reversed by 0.01M cyanide, the concentration used in the measurement of the succinic dehydrogenase by the manometric method, with methylene blue as carrier. This was shown by the fact that the degree of inhibition was the same whether the succinic dehydrogenase activity was measured by this manometric or by the Thunberg method, in which cyanide is not used.

(4) p Chloromercuribenzoate

Heart muscle preparation, treated with sufficient p chloromercuribenzoate to inhibit completely the oxidation of succinate by methylene blue in the absence of potassium cyanide, was reactivated by 80% by the addition of 0.01M cyanide. Strangely, the inhibition was not reversed if cyanide was added to the Barcroft flask after the succinate.

After complete inhibition by 1.5 μ equiv of p chloromercuribenzoate per ml of enzyme preparation, the complete succinic oxidase system was reactivated by 31% with an amount of GSH equivalent to the mercurial used and by 65% with 5 times as much GSH. The succinic dehydrogenase was reactivated to the same extent as the complete system. The enzyme was also readily reactivated (73%) by adding 0.13% denatured globin to the manometric flask in which the enzymic activity was measured*. In marked contrast, however, the enzyme was not in the slightest degree reactivated by BAL in an amount equivalent to 5 times the amount of mercurial used.

C Spectroscopic observations

A normal cytochrome spectrum was obtained when sodium dithionite (Na₂S₂O₄) was added to heart muscle preparation treated with sufficient p aminophenylarsenoxide, o iodosobenzoate or p chloromercuribenzoate to inhibit completely the activity of the succinic oxidase system. The bands did not appear, however, when succinate was used instead of Na₂S₂O₄, showing the absence of an active succinic dehydrogenase. When denatured globin was added to the tube containing succinate and the heart muscle preparation treated with the mercurial, the

* Under the same conditions, the enzyme treated with one third as much arsenical (i.e. 0.5 μ equiv/ml. of heart-muscle preparation) was reactivated by 25% by treatment with globin. The succinic dehydrogenase and the complete succinic oxidase system were reactivated to the same extent.

bands of the cytochromes immediately became visible. Thus the spectroscopic observations are in agreement with the manometric experiments.

DISCUSSION

Inactivation of succinic dehydrogenase by sulphhydryl combining reagents and reactivation by sulphhydryl containing compounds

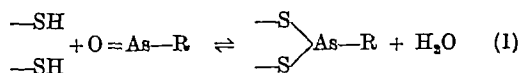
Under the conditions of the experiments described above, the reaction between the enzyme and the arsenical or *o* iodosobenzoate was slow. The slowness of the reaction must be considered when comparing the activity of different arsenicals. Barron, Miller, Bartlett, Meyer & Singer (1947) found that lewisite was much more effective than other organic arsenicals or inorganic arsenite in inhibiting the succinic oxidase system. If all arsenicals combine with the same groups on the enzyme to form a difficultly dissociable complex, one would expect all arsenicals to be equally effective, if they are allowed to react with the pure enzyme for a sufficient length of time. The differences found by Barron, Miller, Bartlett, Meyer & Singer may be due to differences in the rates of combination, not in the capacity for combination, or to differences in the relative rates of combination of the arsenical with the succinic dehydrogenase compared with the rate of combination with other proteins which are present in the enzyme preparation and which protect the enzyme from inactivation. These points require further investigation. It should be noted that the present finding, that the arsenical reacts slowly with the enzyme, is not in agreement with Barron & Singer (1945), who found that the inhibition was complete after 6 min. Many of the findings of the present communication do not agree with those of Barron and his co-workers. This may be due to the different method employed in studying the inhibition and perhaps also to the different enzyme preparation used (Barron & Singer (1945) used a preparation made by treating washed skeletal muscle in a Waring blender with phosphate buffer, it was not purified by acid precipitation). The concentration of the enzyme during treatment with the SH combining reagents in the present studies was about 100 times that used by Barron & Singer (1945).

The finding that GSSG reacts with the enzyme very slowly, and that high concentrations and long incubation times are necessary to obtain an appreciable inhibition, is in agreement with the original observations of Hopkins *et al* (1938). This is probably due to the difficulty of penetration of the large molecule of GSSG (mol wt 612) to the site of the inhibition. Keilm & Hartree (1940) found that disulphides such as tetraethyldithiocarbamyl disulphide and tetrathionate, whose molecular structure resembles that of pyrophosphate which is probably

a competitive inhibitor of succinic dehydrogenase, are much more powerful inhibitors than GSSG possibly because they can penetrate more readily to the active centre of the enzyme which may be the site of the inhibition.

It is customary to express the effectiveness of an inhibitor of an enzyme in terms of the concentration of the reagent which produces a certain inhibition. This implies that the degree of inhibition is, in fact, determined by the concentration of the inhibitor when the enzyme activity is measured and not by the concentration of the enzyme. This is the case when the inhibitor enters into a loose easily reversible combination with the enzyme and the inhibition is reversed by dilution. The inhibitors, with which we are concerned in this paper, do not, however, form such an easily reversible compound, but take part in a chemical reaction which proceeds practically to completion, and the compound formed is not dissociated by subsequent lowering of the concentration of the inhibitor by dilution. The degree of inhibition depends then, not on the final concentration of the inhibitor in the enzyme solution when the activity is measured, but on the ratio of the concentration of the inhibitor to that of the enzyme provided that sufficient time is allowed for the reaction to proceed to completion. The actual concentrations of the reactants will, of course, affect the rate at which this point is reached, and become more important than the ratio of the concentrations when the reaction is very slow, for example, when GSSG is the inhibitor.

If the effect of *o* iodosobenzoate is to oxidize SH groups in the enzyme molecule to S-S, it is not surprising that removal of the inhibitor should have no effect on the inactivated enzyme. It is surprising, however, that, after treatment with the arsenical, the enzyme cannot be reactivated by removing the untreated arsenical and washing with water. It is generally believed that the reaction of the enzyme with the arsenical is

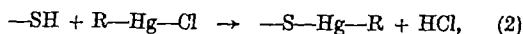


Enzyme Arsenical Enzyme-arsenical compound

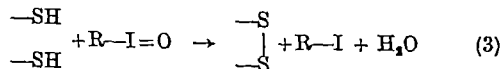
If this is the case, one would expect that removal of the arsenical would cause some dissociation of the inactive enzyme-arsenical compound, but this does not appear to occur. No reactivation was obtained by diluting a partially inactivated enzyme preparation 55 fold even after keeping it 3 hr before measurement of the succinic oxidase activity. The equilibrium of the above reaction must lie far to the right. In this respect, succinic dehydrogenase resembles keratene, Stocken & Thompson (1946*a*) found that 85% of the arsenic combined with the keratene remained after precipitation at pH 4.6 and washing.

p Chloromercuribenzoate differs from the other agents used in several important respects, viz (a) it inactivates the enzyme more rapidly, (b) it does not react with other substances in the enzyme preparation before attacking the enzyme, (c) its action is readily reversed by GSH or denatured globin, but not by BAL, the action of the arsenical is, on the other hand, reversed much more effectively by the dithiol than by the monothiol or globin, and BAL is also rather more effective than GSH in reversing the effect of *o* iodosobenzoate

These differences are probably all connected with the fact that the mercurial combines with only one SH group thus,



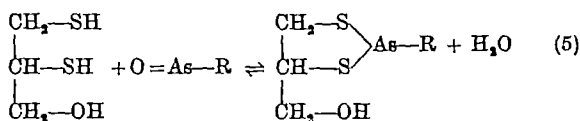
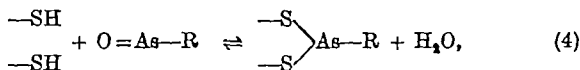
while the arsenical combines with two such groups (either on the same or on different enzyme molecules) as shown in reaction (1) and the oxidizing agent also requires two SH groups to form a disulphide



It is readily understandable that the reaction with one SH group might be more rapid than that with two SH groups. The second point of difference between the mercurial and the other inhibitors probably follows from this difference in the rates of the reaction, if it is assumed that the enzyme preparation contains substances which, unlike the enzyme, are readily attacked by both types of inhibitor. The third point of difference can be explained by assuming that the mercurial readily combines with monothiols, but not with dithiols like BAL. Arsenicals, on the other hand, are known from the work of Stocken & Thompson (1946*b*), extended by Whittaker (1947), to combine much more easily with dithiols, with which they can form stable rings containing the arsenic and the sulphur. GSH and BAL do not differ so markedly in their capacity to reverse inhibition by iodosobenzoate, and the greater activity of BAL in this case may be due to the stronger reducing properties of this compound. Denatured globin behaves, in respect of its ability to reverse inhibition by the mercurial and the arsenical, like a monothiol. Mursky & Anson (1936) have shown that denatured globin, like other denatured proteins, contains a number of reactive SH groups.

It is interesting to note that Barron & Kalnitsky (1947) have found that the inhibiting action on the succinic oxidase system of mercuric chloride, which would be expected to react more readily with dithiols (it gives an insoluble precipitate with BAL) than with monothiols, is reversed much more readily by dithiols than by monothiols.

From the point of view of the position of the final equilibria, the reactivation of the enzyme inhibited by arsenicals can be explained by competition between the BAL and the enzyme for the arsenical according to the equations

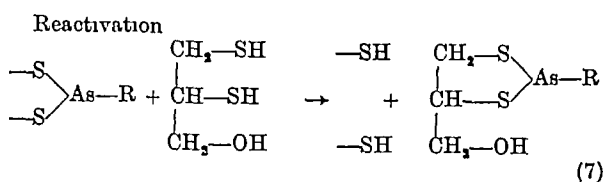
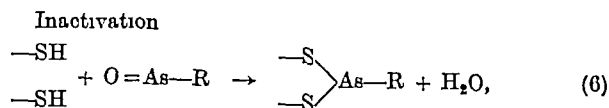


However, such an explanation introduces a kinetic difficulty. It has already been shown that the reaction between the enzyme and the arsenical is slow and that the position of the equilibrium in reaction (4) is far towards the right. It must follow that the reverse reaction (4) is still slower than the forward, consequently no matter how fast the reaction between BAL and the arsenical, the rate of dissociation of the enzyme-arsenical compound would be the reaction limiting the reactivation of the enzyme, which would therefore be very slow. However, contrary to this conclusion, it was found that the reactivation was much more rapid than the inactivation.

This suggests that the above equations do not correctly express the reactions involved. Two possible modifications are as follows.

(a) The process limiting the rate of inactivation is not the actual combination between the enzyme and the arsenical, but the rate of diffusion of the arsenical to the reactive groups of the enzyme. Thus the actual velocities of the forward and reverse reactions (4) may be much greater than indicated by the slowness of the inactivation. According to this view, the relatively rapid reactivation is due to the ease of penetration of BAL to the site of the inhibition, and to the fact that the arsenical-BAL compound diffuses more rapidly than the arsenical. This explanation is supported by the ability of BAL to penetrate rapidly into tissues and thereby combine with lewisite some time after contact of the tissue with the lewisite (Stocken & Thompson, 1946*b*), and by the finding of Peters & Stocken (1947) that the compound between BAL and mapharside (which is very similar to the arsenical used in the present investigation) is more toxic to rats than the arsenical itself, presumably owing to readier penetration of the compound to sites which are dangerous to the animals.

(b) The reactivation is not due to competition between the enzyme and the BAL for the arsenical formed by dissociation of the enzyme-arsenical compound, but essentially irreversible reactions are involved, both in inactivation and reactivation.



Is succinic dehydrogenase a monothiol or a dithiol?

Dithiols were introduced as antidotes in arsenical poisoning by Peters *et al* (1945) Stocken & Thompson (1946*b*) found that the inhibition of the pyruvic oxidase system by arsenicals was completely reversed by dithiols, while monothiol were ineffective, and suggested that this was due to the fact that the arsenical combines with two essential SH groups, closely situated in the enzyme molecule, so as to form a relatively stable cyclic structure. Since the dithiol BAL is also much more effective than GSH in reversing the inhibition of succinic dehydrogenase by *p* aminophenylarsenoxide, it might appear justifiable to conclude that the succinic dehydrogenase molecule also contains two closely situated SH groups.

Before considering this matter, it should be noted that there are important differences between the pyruvic and succinic enzymes with regard to inhibition by arsenicals and reversal by thiols, as has been pointed out by Thompson (1948). Thus, Stocken & Thompson (1946*b*) have shown that monothiol are completely ineffective in preventing or reversing the action of arsenicals on the pyruvic enzyme, whereas Barron, Miller, Bartlett, Meyer & Singer (1947) and the present investigation have shown that GSH is able to reverse partially the inhibition of the succinic enzyme, although it is much less effective than BAL. In addition, it has been shown by Peters, Sinclair & Thompson (1946) that in brain preparations containing both enzymes, the pyruvic is much more susceptible than the succinic enzyme to arsenicals.

It does not necessarily follow from the greater effectiveness of dithiols compared with monothiol in reversing the inhibition by arsenicals that the enzyme is, itself, a dithiol. The weakness of this type of argument is shown by the fact that if applied to the findings with the arsenical it would be concluded that the succinic enzyme was a dithiol, while if applied to the experiments with the mercurial, the conclusion drawn would be that the enzyme was a monothiol. As it is the same enzyme in both cases, the different results obtained must be a property of the inhibitor, not of the enzyme. Since Stocken & Thompson (1946*b*) have shown that dithiols form more stable compounds with the trivalent arsenicals

than do monothiol, it would be expected that the dithiols would be more satisfactory reactivating agents, regardless of the nature of the combination between the arsenical and the enzyme.

Thus it is not permissible to conclude, from the experiments on the reactivation of the enzyme inhibited by arsenicals, that the enzyme contains two SH groups which react with the same arsenic atom. The other findings, in fact, without definitely settling the matter, suggest that it is more likely that the enzyme contains only one SH group necessary for activity, since it is difficult to reconcile otherwise the greatest ease of inhibition by the mercurial with the failure of BAL to reactivate after such inhibition, which shows that the mercurial does not readily react with compounds containing two closely situated SH groups. The finding that BAL in an amount equivalent to the amount of arsenical combined in the enzyme almost completely reactivated the enzyme, showing that the arsenical forms a very much firmer union with the simple dithiol than with the enzyme, is also in agreement with this view. If the enzyme contains only one essential SH group, it is probable that the arsenical and oxidizing agents combine with two SH groups from different molecules, only one of which may be the group on the enzyme necessary for its activity.

Components of the succinic oxidase system inhibited by SH combining reagents

It has been shown that the inhibition of the complete succinic oxidase system by the arsenical can be accounted for by the inhibition of the succinic dehydrogenase. The simplest explanation of the action of arsenicals is that they combine with two SH groups, either both in the same succinic dehydrogenase molecule or on different molecules, and it would be expected that SH groups capable of reacting in this way would be oxidized by oxidizing agents to disulphides. However, the fact that the complete succinic oxidase system is affected to a greater extent than the succinic dehydrogenase shows that the latter is not the only point of attack by oxidizing agents on the system. The curve shown in Fig. 2 indicates that two reactions are proceeding, even at room temperature, and the further oxidation might proceed even more readily at 38°. The other part of the system which is attacked can also be reactivated by treatment with thiols, but with much greater difficulty than is the succinic dehydrogenase. The fact that the action of an oxidizing agent can be reversed by treatment with SH compounds is often taken as an indication that the oxidizing agent has attacked SH groups. However, this does not necessarily follow. Other groupings besides SH groups might be oxidized by oxidizing agents and reduced by subsequent treatment with reducing agents. It seems preferable to consider that SH

groups are attacked only when the enzyme is inhibited by a variety of agents, viz alkylating agents—such as iodoacetate or chloroacetophenone—arsenicals and mercaptide forming agents. In this case, the other portion of the system is not attacked by arsenicals. It has been shown that the cytochrome oxidase activity is not affected by oxidizing agents. It seems possible then, that the oxidizing agents affect, in addition to the succinic dehydrogenase, the component of the succinic oxidase system which links the dehydrogenase to cytochrome c, and, as is discussed elsewhere (Slater, 1949c), is destroyed by coupled oxidation with BAL or GSH. However, the possibility that the oxidizing agents, in addition to their effect on SH groups, have the non specific type of effect on the system discussed by Keilin & Hartree (1949) and Slater (1949d) cannot be excluded.

It is interesting that treatment of the enzyme with GSH, in the absence of air but in the presence of GSSG, increased the inhibition of the succinic oxidase system while the succinic dehydrogenase was completely reactivated. It is possible that GSSG, in the high concentration used in this experiment, may be able to replace oxygen in promoting the destruction by BAL of the component of the system mentioned in the preceding paragraph. However, this point requires further clarification.

It is somewhat surprising that the enzyme, inhibited by oxidizing agents, is not reactivated by potassium cyanide (0.01M), since Walker (1925) showed that cyanide reduces disulphides. Possibly, higher concentrations of cyanide would be effective.

The effect of *p* chloromercuribenzoate is rather complicated. It is clear that it attacks the complete system more readily than the succinic dehydrogenase portion. This is in agreement with the work of Cook & Perisutti (1947) and of Cook, Kreke, McDevitt & Bartlett (1946) on the effect of phenylmercuric nitrate on the succinic oxidase system, although with this reagent these workers found a greater difference between the two inhibitions. *p* Chloromercuribenzoate inhibited cytochrome oxidase, but this is insufficient to account for the greater effect on the complete succinic oxidase system than on the succinic dehydrogenase, since the cytochrome oxidase is not the limiting factor in the succinic oxidase system in the heart muscle preparation. Cook *et al* (1946) also found an inhibition of the cytochrome oxidase activity with phenylmercuric nitrate. They found, in addition, that other enzymes, viz lactic dehydrogenase and catalase, which do not usually react with SH combining reagents, were inhibited by this compound. This suggests that these mercurials have a non specific effect on the entire succinic oxidase system as well as combining with SH groups in the dehydrogenase.

The function of the sulphhydryl groups in succinic dehydrogenase

Since succinate or competitive inhibitors of succinic dehydrogenase (which are assumed to combine with the enzyme at the same point as succinate) protect the enzyme from the action of SH combining reagents, Hopkins & Morgan (1938) suggested that the SH group is at or near the point where succinate attaches itself to the enzyme. However, since succinate was unable to reduce succinic dehydrogenase treated with GSSG, these authors concluded that oxidation of SH to S-S did not play any part in the catalysis of the oxidation of succinate. Potter & Dubois (1943), on the other hand, have ascribed such a catalytic function to the SH group, which is presumed to mediate the transfer of electrons from succinate to cytochrome oxidase, by oscillating between the EnSH and EnS forms. They suggest that the SH group lies between two 'carbonyl affinity groups', which form hydrogen bonds with the carbonyl oxygen atoms of succinic acid. By means of atomic models, Potter & Dubois were able to show that the combination of the type they envisage would result in the hydrogen atoms of succinate being directed towards the SH group, while the hydrogen atoms of malonate would be directed away, thus explaining the fact that malonate, although combining with the enzyme, is not oxidized. Although this theory of Potter & Dubois satisfactorily explains some of the properties of the enzyme, a further examination to see if it can explain the specificity of the enzyme and competitive inhibition by oxaloacetate and pyrophosphate seems to be required.

Unpublished experiments of the author have raised some doubt whether it is even permissible to conclude from the experiments of Hopkins & Morgan (1938) and Potter & Dubois (1943) that the SH group is near the point where the succinate is attached to the enzyme molecule. It was found that succinate and competitive inhibitors will protect the succinic oxidase system from destruction by BAL, although the point of attack on the succinic oxidase system is not even on the succinic dehydrogenase, but on a completely separate component. This suggests that the attachment of succinate or a competitive inhibitor to the succinic dehydrogenase has an effect extending beyond the succinic dehydrogenase molecule itself. It is known that the separate components of the succinic oxidase system must be closely associated with one another, so that each component is easily accessible to those with which it reacts.

SUMMARY

1. Inhibition of the succinic oxidase system by *p* aminophenylarsenoxide, *o* iodosobenzoate and oxidized glutathione is a slow reaction, *p* chloromercuribenzoate reacts much more rapidly.

2 The heart muscle preparation contained substances which protected the succinic oxidase system from small amounts of *p* aminophenylarsenoxide and *o* iodosobenzoate, but not of *p* chloromercuribenzoate

3 *p*-Aminophenylarsenoxide acts on the succinic oxidase system by inhibiting the succinic dehydrogenase. Oxidizing agents (*o* iodosobenzoate, oxidized glutathione and cupric salts) have a more pronounced effect on the succinic oxidase system than on the succinic dehydrogenase, possibly by oxidizing the factor linking succinic dehydrogenase with cytochrome oxidase. *p* Chloromercuribenzoate also affects the succinic oxidase system more than the succinic dehydrogenase, in addition, it has some effect on the cytochrome oxidase. It is likely that inhibition of the succinic oxidase system by *p* chloromercuribenzoate is partly due to a non specific effect on the entire succinic oxidase system.

4 After treatment with sulphhydryl combining reagents, the enzyme was not reactivated by dilution or by precipitation of the enzyme and washing away the excess inhibitor.

5 After inhibition by *p* aminophenylarsenoxide, the enzyme was rapidly reactivated by small concentrations of BAL. Reduced glutathione was less effective and denatured globin only slightly effective as reactivating agents. The succinic dehydrogenase and the succinic oxidase system were reactivated to the same extent.

6 After inhibition by iodosobenzoate, the succinic dehydrogenase was almost completely reactivated by high concentrations of BAL. Reduced glutathione was slightly less effective. The succinic oxidase

system was less readily reactivated than the succinic dehydrogenase.

7 BAL readily reactivated the succinic dehydrogenase after partial inhibition by oxidized glutathione, but increased the inhibition of the succinic oxidase system. Cyanide did not reactivate the enzyme inhibited by oxidized glutathione.

8 Succinic dehydrogenase inhibited by *p* chloromercuribenzoate was largely reactivated by 0.01M cyanide. The succinic oxidase system, after treatment with the mercury compound, was readily reactivated by reduced glutathione and denatured globin, but not by BAL.

9 It is suggested that the action of these inhibitors on the succinic oxidase system be expressed in terms of the amount of inhibitor required to react with a definite amount of the enzyme preparation, not by the concentration of inhibitor required to produce a certain effect.

10 The differences between *p* chloromercuribenzoate and the other inhibitors can partly be explained by the fact that this compound reacts with one sulphhydryl group, while the other compounds require two sulphhydryl groups.

11 The available evidence is not sufficient to decide definitely whether succinic dehydrogenase contains one or two sulphhydryl groups which are necessary for its activity, although one is the more likely.

I would like to thank Prof D Keilin, FRS for his interest and advice, Prof R H S Thompson for providing the BAL, Dr S V Perry for supplying the *p* chloromercuribenzoate and the British Council for a scholarship.

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The Relationship between the Constitution and the Effect of Chemical Compounds on Plant Growth

1 2 PHENOXYETHYLAMINE DERIVATIVES

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(Received 9 February 1949)

The discovery of the selective herbicidal effect of the substituted phenoxyacetic acids (Slade, Templeman & Sexton, 1945, Templeman & Sexton, 1946a) has prompted a further investigation of related compounds, with particular regard to the origin and function of 3 indolylacetic acid in green plants. The auxins of green plants exist only to a limited extent

tration of 3 indolylpyruvic acid produced the same effect, but this was not so with tryptamine. It appears to have been generally assumed, without much direct evidence, that the pathway from tryptophan to 3 indolylacetic acid is the same in plants as in bacteria, proceeding through 3 indolylpyruvic acid by the route indicated in Fig 1. It is

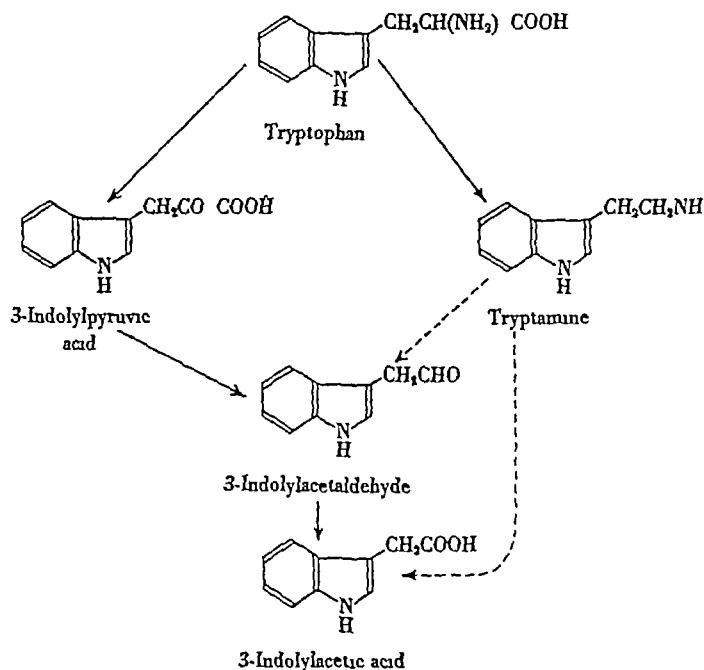


Fig 1 Alternative pathways from tryptophan to β indolylacetic acid

in the free state, the greater portion being present either as complexes, possibly of a proteomaceous nature, or as physiologically inactive precursors. It is generally believed that 3 indolylacetic acid in green plants, as in bacteria, originates from tryptophan, and direct evidence of this has been provided by the finding of Wildman, Fern & Bonner (1947) that infiltration of tryptophan to spinach leaves greatly increased the amount of ether-extractable 'auxin' in a few hours. Oxygen was required in this process, and there was evidence that a carbonyl compound was an intermediate metabolite. Infil-

tration of 3 indolylpyruvic acid produced the same effect, but this was not so with tryptamine. It appears to have been generally assumed, without much direct evidence, that the pathway from tryptophan to 3 indolylacetic acid is the same in plants as in bacteria, proceeding through 3 indolylpyruvic acid by the route indicated in Fig 1. It is theoretically possible, however, that the first step might be decarboxylation to tryptamine, followed by enzymic conversion to 3-indolylacetic acid, perhaps via the aldehyde. It is also possible that more than one route may operate or that different plant species use different routes. Thus, although the experiments of Wildman *et al* (1947) using spinach may be taken as evidence against the tryptamine route, it has been shown by Skoog (1937) that tryptamine can be converted by cut surfaces of *Avena* to a substance having auxin activity. Decarboxylation of amino acids may be inferred to

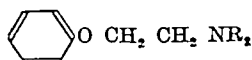
occur in plant tissue as several appropriately constituted amines have been recognized in the free state. Indeed, not only is the molecule of tryptamine discernible in such alkaloids as harmine, physostigmine and dipterine, but it has been found in the free state in certain *Acacia* species (White, 1944). From this it may be concluded that the tryptamine route to 3 indolylacetic acid is worth serious consideration, at least in certain plant species. Finally, it should be added that evidence for the occurrence in plants of 3 indolylacetaldehyde has been produced by Larsen (1944, 1947).

The work here described was based upon the following hypothesis. Plant growth is regulated, perhaps in part, through the maintenance of a critical level or concentration of 3 indolylacetic acid, and this level is maintained in a dynamic system by the balance of anabolic and catabolic processes. Degradation of 3 indolylacetic acid is known to occur in plants, for a degradative enzyme has been isolated from the pea epicotyl (Tang & Bonner, 1947). Any disturbance of this balance will result in hormonal dysfunction which may be revealed by such symptoms as epinasty, organ modification or cessation of growth. It follows from this hypothesis that the normal precursors of 3 indolylacetic acid may, by conversion to the latter compound, produce the same symptoms as 3 indolylacetic acid itself.

Tryptophan itself, although inactive by the *Avena* test for auxin (Link & Eggers, 1943) is, as stated above, convertible to auxin, and it inhibits root growth in cress (Audus & Quastel, 1947). 3 Indolylpyruvic acid is active in the *Avena* test (Kögl & Köstermans, 1935) and so, apparently, is 3 indolylacetaldehyde (Larsen, 1947). The work of Skoog (1937), already referred to, provides one piece of evidence for the conversion of tryptamine to 'auxin'. Our colleague, Dr A. Rhodes of Jealotts Hill Research Station, has found that tryptamine has a marked effect in reducing the root length of germinating cress and a much less marked effect on wheat. We have confirmed this observation (cf. also Audus & Quastel, 1947). Since certain substituted phenoxyacetic acids produce growth effects similar to those of 3 indolylacetic acid, we decided to study the problem further by moving from the indole series to the substituted 2-phenoxyethyl series, where syntheses are much more easy, and by employing the technique of seed germination.

METHODS

Preparation of compounds. The compounds selected for study were the substituted 2-phenoxyethylamines of the general formula



in which the benzene ring contained appropriate substituents and in which R was hydrogen or a hydrocarbon residue. The methods employed were conventional and are given here in outline only, details of the properties of the new compounds being given in Table I, together with such analyses as were deemed desirable to check the purity of individual compounds or of a typical member of a series prepared by a general method.

The amines were made by interaction of the 2-phenoxyethylbromide with excess of NH_3 or the appropriate primary or secondary amine. When the latter was volatile, solutions in methanol under pressure were employed. In the case of 2,2',4'-dichlorophenoxyethylamine a more convenient laboratory method was provided by the decomposition, by means of boiling ethanolic HCl, of the quaternary salt of the phenoxyethylbromide with hexamethylenetetramine.

Quaternary salts were prepared either by quaternization of the tertiary amines with alkyl halide or by reaction of the 2-phenoxyethylbromide with the appropriate tertiary amine. Quaternary salts of 2-phenoxyethylbromides with hexamethylenetetramine were obtained by reaction of the components in hot CHCl_3 solution, from which the crystalline salts separated.

Biological methods. The general requirement for this work was a rapid routine test method which would distinguish readily between active and inactive compounds without necessarily providing accurate quantitative data. Two plant species, rape and oats, were chosen as test objects, since the earlier work of Templeman & Sexton (1946a, b) had shown that several monocotyledonous species were susceptible to isopropyl phenylcarbamate, while some dicotyledonous species were generally the more susceptible to auxin-like herbicides, such as the substituted phenoxyacetic acids. The chosen seeds were oats (*Avena sativa*, var. White Winter) and rape (*Brassica campestris*, var. English Broad Leaved).

The seeds were allowed to germinate on agar slopes in 6 in. \times $\frac{1}{2}$ in. test tubes, each tube containing three rape seeds or two oat seeds. The number of tubes used was 12 for rape or oats at 100 p.p.m. and for rape at 50 p.p.m., 18 for rape at 10 p.p.m. or less and for oats at 50 p.p.m. or less. The tubes were mounted vertically in the dark at room temperature, and the root lengths measured when the rape roots were 5–6 cm long (5–7 days) and the oats 6–7 cm long (8–10 days). Seeds which failed to germinate were rejected, and the average root length ('average control length') of the remaining seeds was measured. With oats, the longest root produced by each seed was measured, since trial experiments had shown this to be reasonably proportionate to the combined lengths of all the roots. In the experimental tubes, the substance under test was incorporated with the agar at a concentration of 100 p.p.m. and greater dilutions were examined subsequently if necessary. The seeds were examined at the same time as the controls, and the effect of the chemical was assessed in the following way. Those substances which caused more than half the roots to be less than 50% of the average control length were characterized as 'very active' and are given two plus signs in the tables. If a substance failed to reduce half the roots to less than 50% of the average control length it was then reassessed on a basis of 80% of the average root length. If more than half the roots were reduced to 80% of the average control length, the substance was marked with one plus sign and characterized as 'active'. If it failed to reduce half the roots to 80% of the average control length, it was characterized as inactive (–).

Table 1 *New compounds synthesized*

Formula	Physical characteristics	Analyses		
			Found (%)	Required (%)
$C_6H_5 \cdot O \cdot CH_2 \cdot CH_2 \cdot NH_2$	Hydrochloride sinters at 180°, m p 210°	(Hydrochloride)	N, 8.0	8.05
$p\text{-Cl} \cdot C_6H_4 \cdot O \cdot CH_2 \cdot CH_2 \cdot NH_2$	Colourless oil, b p 152–156°/15 mm Hydrochloride, m p 228°	(Hydrochloride)	N, 6.8	6.7
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NH_2$	Pale yellow oil, b p 174–176°/15 mm Hydrochloride, m p 188–192°	(Base)	C, 46.8 H, 4.55	46.6 4.4
		(Hydrochloride)	Cl, 44.2 N, 5.8	43.8 5.8
$1,3,4\text{-}C_6H_3Me_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NH_2$	Colourless oil, b p 140°/15 mm Hydrochloride, m p 190°	(Hydrochloride)	N, 7.2	7.0
$1,5,2\text{-}C_6H_3MeCl \cdot O \cdot CH_2 \cdot CH_2 \cdot NH_2$	Colourless oil, b p 154–156°/15 mm Hydrochloride, m p 162–164°	(Hydrochloride)	N, 6.3	6.3
$2,4,5,1\text{-}C_6H_2Cl_3 \cdot O \cdot CH_2 \cdot CH_2 \cdot NH_2$	Colourless oil, b p 170°/15 mm Hydrochloride, m p 237–239°	(Hydrochloride)	C, 35.2 H, 3.35	34.7 3.25
$2\text{-}C_{10}H_7 \cdot O \cdot CH_2 \cdot CH_2 \cdot NH_2$	Hydrochloride chars at 260°	(Hydrochloride)	N, 6.5	6.3
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NHMe$	Colourless oil, b p 160°/15 mm	(Hydrochloride)	N, 5.35	5.45
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMe_2$	Colourless oil, b p 160–164°/12 mm Hydrochloride hygroscopic	—	—	—
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NEt_2$	Liquid, b p 100°/0.15 mm Hydrochloride, m p 127–128°	(Hydrochloride)	N, 4.5	4.7
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NHC_6H_5$	M p 42–44° Hydrochloride decomposed by water, m p 137–140°	(Base)	C, 59.8 H, 4.8 N, 5.4	59.7 4.6 5.0
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMeC_6H_5$	Pale yellow viscous oil, b p 250–260°/15 mm In soluble in dilute HCl	—	Cl, 24.05	24.2
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot N \begin{array}{c} \text{CH}_2 \text{ CH}_2 \\ \diagup \quad \diagdown \\ \text{CH}_2 \text{ CH}_2 \end{array}$	Pale yellow oil, b p 194–196°/15 mm Hydrochloride, m p 158–160°	(Hydrochloride)	N, 4.8	4.5
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot N \begin{array}{c} \text{CH}_2 \text{ CH}_2 \\ \diagup \quad \diagdown \\ \text{CH}_2 \text{ CH}_2 \end{array} O$	Base thick oil, b p 210–212°/15 mm Hydrochloride, m p 86°	(Hydrochloride)	N, 4.45	4.5
$(2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2)_2NH$	Hydrochloride, m p 183–185°	(Hydrochloride)	N, 3.25	3.2
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMe_2 + Br^-$	White crystals, m p 88–89°	—	C, 39.25	40.0
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMe_2Et + I^-$	M p 77–78°	—	—	—
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMe_2(CH_2)_2CH_3 + Br^-$	M p 146–148°	—	—	—
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMe(CH_2)_3CH_3 + Br^-$	M p 194°	—	—	—
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMe_2(CH_2)_5CH_3 + Br^-$	M p 160–162°	—	—	—
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMe_2(CH_2)_6CH_3 + Br^-$	M p 160–162°	—	—	—
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMe_2(CH_2)_{11}CH_3 + Br^-$	M p 120–124°	—	—	—
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NEt_2 + I^-$	—	—	—	—
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NMe \begin{array}{c} \text{CH}_2 \text{ CH}_2 \\ \diagup \quad \diagdown \\ \text{CH}_2 \text{ CH}_2 \end{array} CH_2 + I^-$	M p 158–160°	—	C, 40.7 H, 4.45	40.4 4.8
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot N_4C_6H_{12} + Br^-$ (from hexamethylenetetramine)	M p 164°	—	N, 13.8	13.7
$2,4,1\text{-}C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH \cdot NC_6H_5 + Br^-$ (from pyridine)	M p indefinite (hygroscopic)	—	—	—
$1,3,4\text{-}C_6H_3Me_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot N_4C_6H_{12} + Br^-$ (from hexamethylenetetramine)	M p 176–179°	—	—	—

Table 2 *Herbicidal activity of standard substances*

(The activities in Tables 2-5 are indicated by + and - signs as explained in the text, p 144)

means not examined

Compound	Formula	Concentration (p p m)	Activity	
			Rape	Oats
4-Chloro 2 methylphenoxyacetic acid	2 4 1 C ₆ H ₄ MeCl O CH ₂ COOH	20		++
		10		+
		0.1	++	
2 4 Dichlorophenoxyacetic acid	2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ COOH	0.1	++	
2 Naphthoxyacetic acid	2 C ₁₀ H ₇ O CH ₂ COOH	10	++	
		1	++	
isoPropyl phenylcarbamate	C ₆ H ₅ NH COO CHMe ₂	0.1	-	++
Tryptamine	C ₈ H ₉ N CH ₂ CH ₂ NH ₂	1.0	++	

Basic substances were examined as their hydrochlorides, acidic substances as their Na salts, while the quaternary ammonium compounds were all neutral salts, freely soluble in water at the required concentrations. Using this method of assessment, results obtained with known herbicidal substances are given in Table 2, thereby establishing a reference standard.

RESULTS

Systematic Testing The results of the examination of the various substances are given in Tables 3-5, which are arranged so as to emphasize relationships between structure and activity. All concentrations are expressed in parts/million (p p m).

Table 3 *Effect of substitution in the benzene ring upon the herbicidal activity of 2 phenoxyethylamine*

Compound (R = CH ₂ CH ₂ NH ₂)	Concentration (p p m)	Activity	
		Rape	Oats
C ₆ H ₅ OR	100	-	-
1 4-C ₆ H ₄ (OR) ₂	10	++	-
	2	++	-
	0.1	-	-
2 4 1 C ₆ H ₃ Cl ₂ OR	10	++	-
	2	++	-
	0.1	-	-
1 5 2 C ₆ H ₃ MeCl OR	5	++	-
	1	++	-
	0.1	-	-
1 3 4-C ₆ H ₃ Me ₂ OR	50	++	-
	10	+	-
2 4 5 1 C ₆ H ₃ Cl ₃ OR	50	++	-
	10	-	-
2 C ₁₀ H ₇ OR	10	++	-
	2	-	-
1 C ₁₀ H ₇ OR	50	+	+
4 1 C ₆ H ₄ NO ₂ OR	50	-	-

Preliminary investigations regarding mode of action

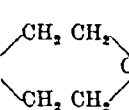
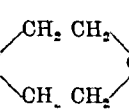
If the working hypothesis is correct, the activity of chlorinated 2-phenoxyethylamine against rape is to

be attributed to its conversion to the corresponding chlorinated phenoxyacetic acid. This might conceivably be brought about through an amine oxidase similar to that found abundantly in animal sources and described by Blaschko, Richter & Schlossmann (1937), and which is inhibited competitively by iso-propylamine derivatives of the type of ephedrine. Tryptamine is known to be a substrate for this enzyme. Accordingly, attempts were made to determine whether the activity of 2 4 dichlorophenoxyethylamine could be antagonized by ephedrine. Ephedrine itself was inactive on both species at 100 p p m and at this strength it failed to antagonize the effect of 5 p p m of 2 4 dichlorophenoxyethylamine.

Veldstra & Havinga (1943) have advocated the hypothesis that substances with an auxin-like activity must be constituted with a suitable nucleus (e.g. indole, naphthalene or substituted benzene) from which an acidic group projects at an angle to the plane of the nucleus. According to this hypothesis, growth regulation by the auxins is a function of the negative pole of the acidic group, which affects ionic transport. It might perhaps be expected then that a similarly constituted molecule, but with a positive instead of a negative pole, would act in a manner antagonistic to that of the naturally functioning auxin or auxin-like substance. Tests with mixtures of 2 4 dichlorophenoxyacetic acid and 2 2' 4' dichlorophenoxyethylamine on rape, however, showed no evidence of antagonism.

In order to obtain further evidence for the hypothesis that the phenoxyethylamines functioned through conversion to the phenoxyacetic acids, certain substances were submitted in a qualitative way to the pea test for auxin activity. This test was conducted as described by Went & Thimann (1937), but since quantitative results were not required, visual inspection was substituted for measurement of angles of curvature. Solutions were examined at a range of concentrations, and the results are given in Table 6.

Table 4 *Effect of N-substitution (formation of secondary and tertiary amines) on the herbicidal activity of substituted 2-phenoxyacetic acids*

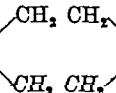
Compound	Concentration (p p m)	Activity	
		Rape	Oats
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NH ₂	2	++	-
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NHMe	50	++	-
	10	-	-
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NMe ₂	100	++	++
	50	++	+
	10	-	-
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NEt ₃	100	++	++
	50	-	+
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NH C ₆ H ₅	100	++	+
	50	++	
	10	+	
	5	-	
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NMe C ₆ H ₅	100	++	-
	50	+	
	10	+	
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ N 	100	++	++
	50	+	++
	20	-	
	10	-	+
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ N 	50	++	++
	10	-	+
(2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂) ₂ NH	50	++	++
	10	-	-
C ₆ H ₅ O CH ₂ CH ₂ NH ₂	100	-	-
C ₆ H ₅ O CH ₂ CH ₂ NH C ₆ H ₅	100	+	+
C ₆ H ₅ O CH ₂ CH ₂ NMe C ₆ H ₅	100	-	+
2 C ₁₀ H ₇ O CH ₂ CH ₂ NH ₂	10	++	-
	2	-	-
2 C ₁₀ H ₇ O CH ₂ CH ₂ NH C ₆ H ₅	100	-	+

DISCUSSION

Considering first the primary amines of Table 3, it is clear that those substituents in the benzene ring which favour activity against dicotyledonous species in the phenoxyacetic acid series (Templeman & Sexton, 1946a) also favour similar activity in the phenoxyethylamine series. The 4 chloro, 2 4 di chloro and 4 chloro 2 methyl derivatives were the most active of the compounds examined, the 2 naphthoxy compound was intermediate in activity, while the unsubstituted compound and the 4 nitro compound were inactive at the concentrations tested. In general, the amines did not appear to be quite as active as the corresponding acids. This finding constitutes evidence in favour of the hypothesis that these substances function in plant growth regulation through conversion to the phenoxyacetic acids. It has been demonstrated experimentally that they do not function as antagonists for the phenoxyacetic acids. Direct biochemical evidence for the hypo-

thesis, however, is lacking. Blaschko *et al* (1937) failed to find amine oxidase in certain green plants (seeds, tubers and leaves), but it does not follow from this that the enzyme is absent from germinating rape seed. It is possible, however, that a different enzyme system may be responsible for the degradation of tryptamine or the phenoxyethylamines in green plants, and this would account for our failure to antagonize the effect of 2 4-dichlorophenoxyethylamine by the amine oxidase inhibitor, ephedrine. Furthermore, the antagonism experiment would involve the question of the cell permeability of ephedrine, about which nothing is known. The failure to antagonize the effects of 2 4 dichloro phenoxyethylamine in this manner does not therefore invalidate the hypothesis concerning its mode of action. Two further facts in support are to be noted. First, the activity of 2 4-dichlorophenoxyethylamine in the pea test is of a similar nature to that of 2 4 dichlorophenoxyacetic acid though it differs quantitatively. Secondly, a preliminary observation in the

Table 5 *Herbicidal activity of quaternary derivatives of substituted 2 phenoxyethylamines*

Compound	Concentration (p p m)	Activity	
		Rape	Oats
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NMe ₃ ⁺ Br ⁻	50	-	+
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NMe ₂ Et ⁺ I ⁻	50	+	++
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NMe ₂ (CH ₂) ₂ CH ₃ ⁺ Br ⁻	50	++	+
	10	+	
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NMe ₂ (CH ₂) ₃ CH ₃ ⁺ Br ⁻	50	++	+
	10	++	
	5	+	
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NMe ₂ (CH ₂) ₆ CH ₃ ⁺ Br ⁻	50	++	-
	10	++	
	5	+	
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NMe ₂ (CH ₂) ₁₁ CH ₃ ⁺ Br ⁻	50	-	-
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NMe ₂  CH ₃ ⁺ I ⁻	50	+	++
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NEt ₃ ⁺ I ⁻	50	++	++
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ N ₄ C ₆ H ₁₂ ⁺ Br ⁻ (a)	50	++	+
	10	++	-
	5	++	
2 4 1 C ₆ H ₃ Cl ₂ O CH ₂ CH ₂ NC ₅ H ₅ ⁺ Br ⁻ (b)	20	+	++
	10	-	-
2 4 1 C ₆ H ₃ Me ₂ O CH ₂ CH ₂ N ₄ C ₆ H ₁₂ ⁺ Br ⁻ (a)	50	++	+
	20	++	-
	10	-	
p Cl C ₆ H ₄ O CH ₂ CH ₂ N ₄ C ₆ H ₁₂ ⁺ Br ⁻ (a)	50		++
	10	++	
2-C ₁₀ H ₇ O CH ₂ CH ₂ N ₄ C ₆ H ₁₂ ⁺ Br ⁻ (a)	100		+
	50	+	

(a) From hexamethylene tetramine

(b) From pyridine

field indicated a similar physiological response of growing buttercups to both the amine and the acid. Direct experiments on the conversion of the amine to the acid in plant tissue or by plant extracts have not yet been made.

Table 6 *Results of the pea test for auxin activity*

Compound	Concentration (p p m)	Effect observed
2 4-Dichlorophenoxy acetic acid	1	Equal to 3 indolylacetic acid at 5 p p m
2 4-Dichlorophenoxy ethylamine	10	Equal to 3 indolylacetic acid at 1 p p m
Quaternary compound of hexamethylenetetramine with 2 4-dichloro phenoxyethyl bromide	100	Equal to 3 indolylacetic acid at 5 p p m

Turning now to the first three compounds in Table 4 it is seen that substitution of the amino hydrogen atoms by methyl groups decreases the activity towards rape and increases the susceptibility of oats. The fully methylated compound is apparently much more active against oats than is

2 4 dichlorophenoxyacetic acid. This can be interpreted in more than one way. The relative insusceptibility of oats to the phenoxyacetic acid or the primary phenoxyethylamines might be due to the failure of these compounds to penetrate in adequate amounts to the appropriate site of action, methylation might assist such penetration, thus causing the phenoxyacetic acid to be liberated at a place which would otherwise be inaccessible to this molecule. The oat plant can, of course, respond to the phenoxyacetic acids under appropriate circumstances, for they are active in the standard *Avena* coleoptile test for auxins. It is possible, though perhaps not very likely, that methylation has the reverse effect on permeability in rape, so that the activity against germinating rape seed is reduced. This question cannot be answered until much more is known of the permeability of different plant species and tissues to organic compounds. Another possibility is that the secondary and tertiary amines function by an entirely different mechanism in which the greater susceptibility of oats is inherent. On the whole, the results given in Table 4 show that with the secondary and tertiary amines generally, there is much less

difference in the susceptibility of the two species than with the primary amines. This caused us to take the further step of examining quaternary compounds.

Amongst the quaternary compounds of Table 5, the 2,4-dichloro series will be considered first. It can be seen that as the molecular weight in the series of *n*-alkyl compounds rises, the activity against rape increases to C_6 and C_8 , but the C_{12} compound is inactive. This is the familiar peak effect in an homologous series which is found in many relationships between chemical constitution and biological activity. Against oats there appears to be a maximum at C_8 , though the result with the C_6 compound is anomalous. In view of the possibility that these quaternary compounds may be functioning by a mechanism bearing no particular relation to the chlorinated phenoxy nucleus, a general investigation of the activity of quaternary salts is now in progress and will be reported separately.

The quaternary compounds of hexamethylenetetramine call for special comment. Such compounds are known to decompose in boiling neutral aqueous solution with generation of aldehydes according to an oxidation-reduction reaction which has recently been discussed by Angyal & Rassack (1948), and we have evidence of decomposition at room temperature. Attempts to convert the hexamethylenetetramine derivative of 2,4-dichlorophenoxyethyl bromide into the aldehyde met with mixed success. While boiling with water definitely gave rise to some aldehydic material, as judged by the formation of a bisulphite compound, the aldehyde itself could not be isolated and appears to be unstable, compare the instability of phenoxyacetaldehyde (Pomeranz, 1894).

Under acidic conditions these hexamethylenetetramine derivatives are decomposed to the primary amines. This reaction has been found to proceed well in the case of the 2,4-dichlorophenoxy derivatives. Thus it may be argued that the hexamethylene tetramine compounds are potential precursors of the

phenoxyacetic acids in living systems. In accordance with this conception, both the 2,2',4,4'-dichlorophenoxyethyl and 2,2',4,4'-dimethylphenoxyethyl compounds of hexamethylenetetramine are active against rape at high dilution, and indeed the former has been shown to produce the characteristic response of the substituted phenoxyacetic acids with growing buttercups. It is possible therefore that these compounds owe their activity against oats to their quaternary structure, but that their high activity against rape is due to their conversion to the acids within the living organism.

(Note added 8 April 1949.) Since this paper was submitted for publication, confirmation of our hypothesis that indolylacetic acid can arise in plants from tryptophan either through indolyl pyruvic acid or through tryptamine has been presented by Gordon & Nieva (1949). These authors have demonstrated the operation of the two alternative pathways in leaves of pineapple.

SUMMARY

1 The activity of substituted β -phenoxyethylamines against germinating oat and rape seed has been studied in the laboratory.

2 In the case of the primary amines, selective activity against rape is associated with the same benzene substituents as cause activity in the phenoxyacetic acid series.

3 Substitution in the amino group tends to alter the relative susceptibility of the two species, and there is evidence that the quaternary salt derivatives operate by a different mechanism.

4 Quaternary derivatives of appropriately substituted 2-phenoxyethyl bromides with hexamethylenetetramine are particularly active against rape.

5 The results are discussed in relation to a hypothesis that phenoxyethylamines are converted by the living plant to the phenoxyacetic acids.

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The Reduction of Gastric Acidity by Back-Diffusion of Hydrogen Ions Through the Mucosa*

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(Received 4 January 1949)

It is generally believed that the hydrochloric acid solution secreted by the oxyntic cells is isotonic with the blood, and that the reduction of the acidity observed in the gastric juice is due to subsequent dilution and neutralization by other secretions (see Babkin, 1944) Teorell (1933), using anaesthetized or decerebrate cats whose stomachs were tied at both ends, observed a rapid decrease in the acidity of hydrochloric acid solutions injected into the stomach, without a corresponding reduction of the volume of the injected fluid. From these results, and from model experiments with artificial membranes, he inferred that the reduction of the acidity of the gastric juice was due mainly to the diffusion of H^+ ions through the gastric mucosa into the blood. However, diffusion of H^+ ions through living gastric mucosa has not so far been demonstrated, and this paper describes an attempt to investigate the diffusion of H^+ ions through isolated amphibian gastric mucosa. The results show that H^+ ions can diffuse through surviving mucosa and that the rates of diffusion are linearly related to the H^+ ion concentration of the solution in contact with the secretory side of the tissue (see Teorell, 1933, 1939).

METHODS

Saline media The salines, isotonic with frog blood, described by Davies & Turner (1949) were used. These were (a) a bicarbonate saline, in equilibrium with 5% CO_2 + 95% O_2 , or with 5% CO_2 + 95% N_2 , and (b) a phosphate saline, in equilibrium with 100% O_2 . All salines contained 0.2% glucose.

Apparatus The apparatus described by Davies & Turner (1949) was used in experiments with 'open sheets' of mucosa. It consists of a constant pressure manometer, a modified Warburg cup B and a special hollow stopper S to which a graduated capillary side tube G, is attached. A diagram of the apparatus is shown in Fig. 1 (Davies & Turner, 1949). Experiments with 'tied' mucosa were carried out in standard conical Warburg cups and manometers.

Analysis of solutions All titrations and measurements of pH were done with the semi micro electrometric titration unit described by Davies & Longmuir (1948).

* A part of this work has been communicated to the Biochemical Society (Turner, 1949).

Material Toads (*Bufo bufo bufo* L.) and frogs (*Rana temporaria temporaria* L.) were captured locally and kept in glass tanks. An excess of worms was provided for food. The animal was killed and the stomach removed and washed with saline. The tube of mucosa was isolated by removing the muscle layer and prepared in two ways, to give either open sheets of mucosa or tied mucosa.

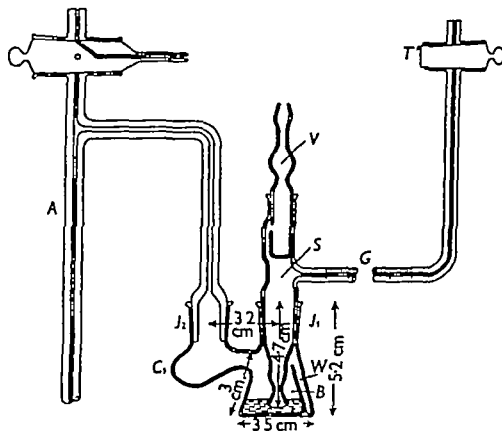


Fig. 1. Vessel B, hollow stopper S, with graduated side tube G, and part of manometer A. Capacity of B, 25 ml, capacity of side arms C_1 and C_2 (C_2 is not shown), 1 ml. Outer diameter of G, 0.6 cm, bore 0.1 cm, length 15 cm, graduation 1 division = 1 μ l. The mucosa is tied to the base of S (diameter 0.9 cm).

Open sheets of mucosa

The tube was opened by cutting along the lesser curvature (Davies, 1948) and the open sheet was tied with silk thread to the open base of a stopper S (Fig. 1) with the secretory side upwards. The secretory surface of the mucosa was covered with a solution of HCl which was pipetted into stopper S. The solutions contained 0.1, 0.05, 0.02 and 0.01 N HCl and sufficient NaCl to make them isotonic with frog blood (0.12 M for univalent-univalent electrolytes). Stopper S and part of the graduated capillary side tube G were then filled with liquid paraffin, and the stopper was closed by inserting the tap stopper V (Fig. 1). The unit was placed in the main compartment of the vessel B, which contained 5 ml of nutrient saline. The vessel was attached to the manometer and, after gassing, was placed in a water bath and shaken at 25.0°. An identical unit in which the mucosa was replaced by a rubber or plastic membrane was used as a thermo-

barometer. The manometers were of the constant pressure type, and the pressure inside the system could be adjusted so that a known resultant pressure was applied to the mucosa throughout the experiment. The pressure was measured in mm. manometric fluid (10,000 mm manometric fluid = 1 atm) (The manometric fluid in use in this laboratory, a modified Brodie solution, is prepared in the following way: 17.5 g Na_2SO_4 (anhydrous), 5.0 g sodium glycocholate, and 0.5 g methyl violet, are dissolved in 500 ml distilled water, 2-3 drops of capryl alcohol are added to prevent foaming. The solution is filtered before use.)

When pressure was applied in the direction of normal secretion (from the nutrient to the secretory side of the mucosa) it was called a positive pressure (e.g. +20 mm manometric fluid), conversely, pressures opposing the direction of normal secretion were indicated by a negative sign (see Davies & Turner, 1949).

Readings were taken at 15 min intervals and the changes in the volume of the gas in the manometer and the volume of fluid in stopper *S* recorded. The following measurements were made: (1) the rates of respiration (Q_{O_2}) and of fluid transport across the mucosa (q_{H_2O}) were measured in phosphate saline, gassed with 100% O_2 , CO_2 being absorbed by $NaOH$ in a glass pocket fused to the wall of vessel *B* (Fig. 1), (2) the rates of acid secretion (Q_{HCl}), or of diffusion of H^+ ions ($q_{CO_2}^{H^+}$) and the q_{H_2O} , were measured in bicarbonate saline gassed with 5% CO_2 + 95% O_2 , (3) the sum of $q_{CO_2}^{H^+}$ and the Q_{CO_2} , and the q_{H_2O} , were measured in bicarbonate saline gassed with 5% CO_2 + 95% N_2 . One mucosa was needed for each set of measurements.

Calculation of results. Since the secretion of liquid into stopper *S* during any period resulted in a loss of liquid from the cup *B*, allowance had to be made for this reduction of volume when calculating the gas exchanges. Thus, when the change of volume in the manometer was $-10 \mu l$, while the increase in the volume of liquid in the graduated capillary tube *G* was $+2 \mu l$, the uptake of gas was actually $-10 + 2 = -8 \mu l$. This was multiplied by a constant which depended on the gas used to give the volume of gas at $N T P$ (For theory of the apparatus see Davies & Turner, 1949).

Atropine (final concentration $10^{-3} M$) was added to the saline to reduce the contractions of the muscularis mucosae (Davies & Turner, 1949). Nevertheless, contractions occurred in 30% of the experiments, in these no reliable measurements of the rates of fluid secretion could be made. The measurement of the rates of gaseous exchanges was, however, not affected, since a contraction of the mucosa, although it produced an apparent uptake of gas by reducing the gas space of the manometer at constant pressure, also caused an apparent increase of the volume of fluid. Since allowance was made for these changes in calculating the gas exchanges the two effects cancelled out. Histamine (final concentration $5 \times 10^{-5} M$) and thiocyanate (final concentration 0.01 M) were added from the side arms.

After the experiment the mucosa was cut along the silk ligature, removed in two portions (circle and rim), washed in distilled water and dried overnight at 110° . The dry weight of the circular portion was used in the calculation of the $q_{CO_2}^{H^+}$, Q_{HCl} and q_{H_2O} . The Q_{O_2} was calculated by using the sum of the dry weight of the two portions. The $q_{CO_2}^{H^+}$, Q_{HCl} and Q_{O_2} are defined as μl gas at $N T P$ / mg dry wt / hr, the q_{H_2O} as μl transported fluid / mg dry wt / hr. (In the case of $q_{CO_2}^{H^+}$, $1 g$ ion $H^+ = 22.4 l$.)

In bicarbonate saline, the CO_2 uptake by gastric mucosa in excess of its basal gaseous exchanges is equivalent to the amount of acid produced (Davies, 1948). In this paper, $-Q_{CO_2}$ denotes the rate of uptake of CO_2 in excess of the basal gaseous exchange of the tissue. Similarly, $+Q_{CO_2}$ does not denote the rate of evolution of respiratory CO_2 , but the rate of extra CO_2 evolution, due to liberation of gas from the bicarbonate saline by H^+ ions diffusing into it from the HCl solution across the mucosa. Since the basal gaseous exchanges result in only a small uptake of gas (about $5 \mu l/hr$, i.e. less than the experimental error), this was neglected. Therefore $-Q_{CO_2} = Q_{HCl}$, and $+Q_{CO_2} = q_{CO_2}^{H^+}$.

Tied mucosa

The tube of gastric mucosa was tied with silk thread at both ends and incubated in Warburg cups containing 4 ml of bicarbonate saline gassed with 5% CO_2 + 95% O_2 at 25° (Davies, 1948). The resultant of O_2 uptake and CO_2 output was measured with Warburg manometers. Secretion of HCl was induced by addition of histamine to a final concentration of $5 \times 10^{-5} M$. Readings were continued for periods up to 10 hr to see whether the acid secreted by the mucosa itself would diffuse out of the bag in which it had accumulated during the secretory phase. At the end of the incubation period the distended bag was blotted and weighed. It was cut open, the contents collected in a sample tube and the empty bag reweighed. The weight of the secreted fluid was given by the difference of the wet weight of the bag before and after incubation. The silk was removed and the tissue washed in distilled water and dried overnight at 110° .

RESULTS

Diffusion of H^+ ions through sheets of mucosa

Aerobic experiments. In these, a sheet of gastric mucosa separated a solution of HCl in stopper *S* from bicarbonate saline in the vessel *B* (see Fig. 1). In all experiments with non-secreting mucosa in contact with 0.1, 0.05 and 0.02 N HCl solutions, evolution of CO_2 was observed while the volume of the HCl solution remained approximately constant, indicating that H^+ ions diffused across the membrane and liberated CO_2 from the bicarbonate solution. The output of CO_2 decreased gradually, and when the acidity of the remaining HCl solution was plotted against time, an exponential curve was obtained (Fig. 2). Titration of samples of the HCl solutions recovered after the experiments showed a reduction in the acidity which was equivalent to the amount of CO_2 evolved during the experiments.

The equation used by Teorell (1947)

$$H = H_0 e^{-ct/p}$$

expresses the decrease in the acidity of the HCl solution with time, where H_0 = initial acidity of HCl solution (mmol or μl), H = acidity at time t (min), p = volume of solution (ml) and c = permeability coefficient (a constant). In nine aerobic experiments with 0.1 N HCl , c for 1.3 cm^2 sheets of gastric mucosa of average thickness 0.3 mm was from

3.2×10^{-4} to 13.0×10^{-4} ml min⁻¹ (average 9.0×10^{-4} , standard deviation 2.7×10^{-4}). Similarly, in seven experiments with 0.05, 0.02 and 0.01N HCl, c was from 2.2×10^{-4} to 11.0×10^{-4} ml min⁻¹ (average 7.0×10^{-4} , standard deviation 2.9×10^{-4}). There was, therefore, no significant difference between the values of c obtained with HCl solutions of different concentrations. Although all mucosae were tied to the base of stoppers of about 1.0 cm² cross sectional area, they bulged to varying degrees, depending on the magnitude of the applied pressure and the state of contraction of the muscularis mucosae. This resulted in a slight variation in the surface areas of the mucosae, which, together with their different thicknesses (apart from individual variations the thickness of the mucosa also depends on the state of contraction), probably accounts for the relatively wide range of the values of c . In any one non-secreting mucosa, however, c was constant. This is illustrated in Fig. 2.

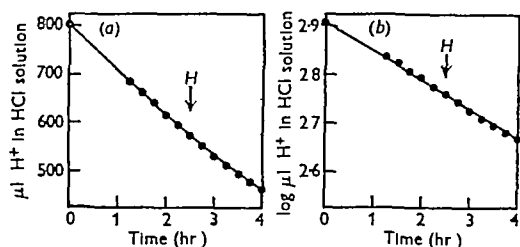


Fig. 2. Diffusion of H^+ ions through non-secreting gastric mucosa. Secretory side of mucosa in contact with 0.4 ml 0.09N HCl. Incubated at 25.0° in bicarbonate saline containing 0.2% glucose, gassed with 5% CO_2 + 95% O_2 . Histamine added from side arm at arrow marked H (final concentration $5 \times 10^{-5}M$). Pressure -20 mm manometric fluid. Dry weight of secretory portion of mucosa 11.3 mg. In (a) the μl H^+ remaining in the HCl solution, and in (b) $\log \mu l$ H^+ , are plotted against time.

The initial rate of CO_2 evolution depended only on the concentration of the HCl solution, and a comparison of results from experiments with 0.05, 0.02 and 0.01N-HCl showed that the initial amounts of CO_2 evolved were approximately $\frac{1}{2}$, $\frac{1}{3}$ and $\frac{1}{10}$ respectively of the amount evolved from 0.1N HCl. The subsequent rate of CO_2 evolution followed the exponential equation

The diffusion coefficient D (cm²sec⁻¹) was calculated from c using the relation $c = D \frac{A}{h} \times 60$, where A = area of the membrane (cm²) and h = its thickness (cm) (Teorell, 1947), or directly from the formula

$$D = \frac{h \times Q_{ml}}{A \times t},$$

where Q_{ml} = the number of ml of the HCl solution which contain an amount of H^+ ions equal to the amount which has diffused, and t = the time in sec (see Bull, 1943). The average diffusion coefficient D

for the above sixteen experiments was 0.03×10^{-5} cm²sec⁻¹ (D for 0.1N-HCl in aqueous solution at 19.3° is 2.5×10^{-5} cm²sec⁻¹, Thover, 1902).

The resultant pressures applied to the nutrient side of these mucosae varied from -40 to +20 mm manometric fluid. Within this range the rate of diffusion of H^+ ions through aerobic mucosa was not dependent on the applied pressure.

Anaerobic experiments. When the pressure was equal on both sides of the mucosa (applied pressure = 0), the permeability coefficient c was of the same order in anaerobic experiments as aerobically. A complication arose when pressure was applied, since, under anaerobic conditions, gastric mucosa allows fluid to traverse it in the direction of applied pressure (Davies & Turner, 1949). On application of negative pressure some acid solution was forced through the tissue and the evolution of CO_2 from the bicarbonate of the medium was increased. The amount of CO_2 evolved by diffusing H^+ ions only was calculated by subtracting from the total amount of CO_2 evolved the equivalent of the amount of H^+ ions contained in the volume of solution which had been forced through the mucosa during the same period. This correction was made on the assumption that the fluid traversing the membrane contained the same concentration of H^+ ions as the HCl solution in contact with the tissue. The permeability coefficient c at negative pressures calculated in this way did not differ from the value determined for the same mucosa in the absence of applied pressure. Positive pressure forced neutral fluid from the nutrient to the secretory side, the rate of CO_2 evolution was reduced probably because the neutral fluid, which streamed through the mucosa in the direction opposing diffusion, diluted the HCl in contact with the tissue. This resulted in lower values for c .

Similar results were obtained in control experiments in which 'dead' frog gastric mucosa (tissue partly dried in air and then steeped in 0.01M KCN for 10 min), dead pig bladder (dried tissue soaked in saline for several days before use) and sheets of cellophane were used as the membrane separating the HCl and bicarbonate solutions. For 'dead' mucosa the diffusion coefficient D was 0.02×10^{-5} cm²sec⁻¹ (one experiment), for dead pig bladder D was 0.15×10^{-5} cm²sec⁻¹.

Secretion and diffusion. Since HCl secretion by a gastric mucosa is accompanied by the uptake of an equivalent amount of CO_2 (Davies, 1948) it was expected that the output of CO_2 , due to diffusion of H^+ ions from the secretory to the nutrient side of the tissue, would be reduced by the onset of secretion. Histamine was added from the side arm of the vessel after a steady evolution of CO_2 had been observed. Diminution of the rate of CO_2 evolution was not observed when 0.1N HCl was in contact with the

secretory side of the tissue However, with 0.05N-HCl, stimulation by histamine resulted in a decrease of the CO₂ evolution In Fig 3 the reduction of CO₂

secretion of HCl (Davenport, 1940, Crane, Davies & Longmuir, 1946), was added to a final concentration of 0.01M the original rate of CO₂ evolution was restored

When 0.02N HCl was allowed to diffuse through a resting mucosa, the evolution of CO₂ stopped after stimulation by histamine, and the subsequent uptake of CO₂ showed that acid was being secreted by the tissue (Fig 4) The acidity of the secreted HCl solution, calculated from the rate of fluid production and uptake of CO₂, was low If, however, the average rate of CO₂ evolution ($q_{CO_2}^{H+}$), observed before the addition of histamine, was added to the Q_{HCl} , the acidity of the secreted HCl solution was estimated to be between 0.1 and 0.12N (Table 1)

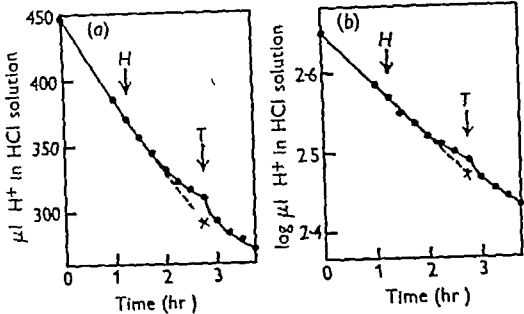


Fig 3 Diffusion of H⁺ ions through gastric mucosa Effect of stimulation by histamine Secretory side of mucosa in contact with 0.4 ml 0.05N HCl Incubated at 25.0° in bicarbonate saline containing 0.2% glucose, gassed with 5% CO₂+95% O₂ In (a) the μl H⁺ in the HCl solution, and in (b) log μl H⁺, are plotted against time Histamine added at arrow marked H (final concentration 5 × 10⁻⁵M), thiocyanate added at arrow marked T (final concentration 0.01M) Pressure -30 mm. manometric fluid Dry weight of secretory portion of mucosa 5.3 mg The broken line indicates the (calculated) slope of the curve if there had been no secretory response to histamine

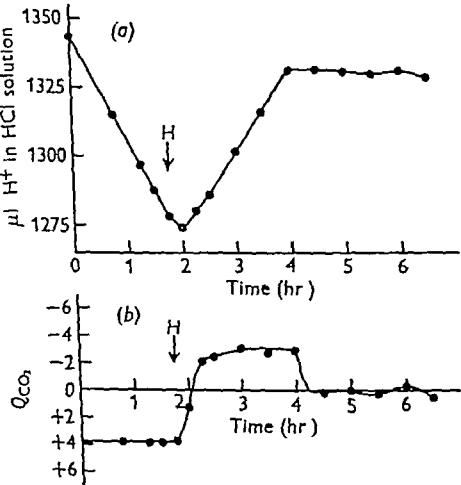


Fig 4 Diffusion and secretion of H⁺ ions Secretory side of mucosa in contact with 3 ml 0.02N HCl Incubated at 25.0° in bicarbonate saline containing 0.2% glucose, gassed with 5% CO₂+95% O₂ In (a) the μl H⁺ in the HCl solution are plotted against time in (b) the rates of diffusion and secretion of H⁺ ions are given for comparison Histamine added at arrow H (final concentration 5 × 10⁻⁵M) Pressure -40 mm manometric fluid Dry weight of secretory portion of mucosa 10.3 mg

evolution during the period 1 hr 45 min to 2 hr 45 min (about 17 μl) corresponds to an average Q_{HCl} of about 3 When thiocyanate, which inhibits

Table 1 Back diffusion and secretion of H⁺ ions

(Secretory side of sheet of mucosa in contact with 0.5 ml 0.02N HCl, nutrient side with bicarbonate saline, gassed with 5% CO₂+95% O₂, and containing 0.2% glucose Histamine added to nutrient saline from side arm at 0 hr 45 min Dry weight of secretory portion of mucosa 7.0 mg Pressure -10 mm manometric fluid + $Q_{CO_2} = q_{CO_2}^{H+}$, - $Q_{CO_2} = Q_{HCl}$)

Time (hr)	Observed (μl/mg dry wt/hr)	Apparent molarity of secreted HCl	Calculated molarity of secreted HCl
	$Q_{CO_2} + q_{CO_2}^{H+}$	Q_{HCl}	$Q_{HCl} + q_{CO_2}^{H+}$
1	+2.4	0	—
2	-1.6	0.06	4.0
3	-2.2	0.06	4.6
4	-1.6	0.045	4.0
5	-0.9	0.03	3.3

Addition of HCl solutions to secreting mucosa In the experiments described above, solutions of HCl were placed in contact with the secretory side of freshly dissected gastric mucosa Although the mucosae were undoubtedly alive, the diffusion rates were high and often exceeded the fastest secretory rate so far observed ($Q_{HCl}=10$), and it seemed probable that the tissue had been damaged In modified experiments isotonic sodium chloride solution was placed in contact with the secretory side of the mucosa, the latter was incubated with bicarbonate saline, and secretion was induced by histamine When the secretory response was established, as shown by the uptake of CO₂, the apparatus was opened and strong HCl added to the NaCl in contact with the mucosa to give a 0.1 or 0.12N HCl solution The acid was thus added after the mucosa had recovered from the trauma due to manipulation and had covered itself with mucus The uptake of CO₂ continued for varying periods at a reduced rate and was then followed by evolution of CO₂ In these experiments the diffusion coefficient D was from 0.01×10^{-5} to 0.015×10^{-5} cm²sec⁻¹

Table 2 Back diffusion of secreted H^+ ions

(Tied mucosae were incubated in bicarbonate saline containing 0.2% glucose, gassed with 5% CO_2 + 95% O_2 . Histamine was added to a concentration of $5 \times 10^{-5} M$. At the end of the experiments on mucosae *D* and *E*, thiocyanate was added to a concentration of 0.02 M . The tubes were weighed (a) before, (b) after incubation, and (c) after emptying. The difference (b) - (a) = weight of secretion, (b) - (c) = weight of contents. The difference between the weight of contents and secretion corresponds to the difference in the weight of the empty tissue before and after incubation, and is mainly due to extrusion of mucus and some tissue degradation. The high pH values observed at the end of the experiments are partly due to buffering of the HCl by cellular debris.)

Mucosa	Dry wt of mucosa (mg)	Area of mucosa (cm. ²)	Wet wt. of contents (mg)	Wet wt of secretion (mg)	Duration of secretory period (hr)	H^+ produced (μ l.)	Molarity of H^+ in secretion at end of secretory period	CO_2 evolved (μ l.)	Duration of back diffusion (hr)	pH of contents at end of exp	Average (μ l/mg dry wt/hr)			State of tissue
											Q_{HCl}	Q_{H_2O}	$Q_{CO_2}^{H^+}$	
A	43.2	5.64	667	590	4.0	828	0.063	232	3	2.9	4.8	3.4	1.8	Undamaged
B	15.0	2.54	—	—	3.5	286	—	57 (+06 leak)	1	2.6	5.4	—	3.1	1 perforation, 2 small ulcers at 6.5 hr
C	12.6	1.85	—	—	4.0	242	—	(54 leak)	—	1.75	4.8	—	—	1 perforation at 5 hr
D	41.0	5.25	456.5	430	9.25	650	0.068	180	1	1.84	1.7	1.1	—	Undamaged
E	39.0	5.45	215	185	8.5	216	0.052	36	—	3.48	0.7	0.6	—	Undamaged

Experiments in phosphate saline With phosphate saline, gassed with 100% O_2 , as the nutrient medium, the rate of diffusion of H^+ ions could not be observed manometrically. The overall reduction in acidity was determined by titration of samples of the HCl solution recovered after incubation, and it was of the same order as in corresponding experiments in bicarbonate saline. Stimulation by histamine resulted in an increase of the Q_{O_2} (see Davies, 1948). In two experiments both mucosae, which were in contact with 0.1 N HCl, showed signs of damage 0.5 and 1 hr respectively after their Q_{O_2} values had increased, their Q_{O_2} fell to about 50% of its maximum value and the mucosae leaked. One mucosa, in contact with 0.1 N HCl to which histamine was not added, had an average Q_{O_2} of -1.5 throughout a 3.5 hr experiment and appeared undamaged. In the presence of more dilute HCl solutions, the Q_{O_2} increased after stimulation by histamine and remained steady during experiments of 3-4 hr duration without damage to the tissue.

Diffusion of secreted H^+ ions through tied mucosa

Tied gastric mucosae were incubated in the bicarbonate saline, gassed with 5% CO_2 + 95% O_2 , in Warburg cups and stimulated by addition of histamine (Davies, 1948). The experiments were extended over 10 hr since it was expected that some of the acid accumulated in the bags might diffuse out at the end of the secretory phase. Typical results are given in Tables 2 and 3. Evolution of CO_2 due to back diffusion of H^+ ions was observed in three out of ten experiments. Four highly active mucosae (max $Q_{HCl} > 5$) were seen to have perforated ulcers when CO_2 evolution started (Davies & Longmuir, 1948, reported and discussed one case of ulcer formation in bicarbonate saline). However, mucosae with even larger perforations could continue to take

up CO_2 , unless some of the contents were forced out by the pressure in the bag (Table 3, mucosa *C*). This may be due to buffering of the acid contents by cell debris from the damaged area, with a consequent reduction of the rate of back diffusion. On the other hand, mucosae of low secretory activity could maintain a steady rate of CO_2 uptake for many hours with no observable back diffusion (Table 3, *D* and *E*).

Table 3 Time course of gas exchanges of experiments summarized in Table 2

Time (hr min)	Q_{CO_2}				
	A	B	C	D	E
0 00	Histamine added from side arm			Histamine in nutrient medium	
0 30					
1 00	-5.3	—	-4.8	-2.1	-0.3
1 30	-6.5	-5.3	-5.6	-1.9	-0.7
2 00	-6.5	-6.9	-5.4	-1.8	-0.8
2 30	-6.3	-7.2	-5.9	-2.0	-1.0
3 00	-5.9	-6.4	-4.9	-1.9	-1.5
3 30	-4.9	-6.0	-4.9	-2.0	-1.0
4 00	-2.7	-4.1	-3.8	-1.9	-1.1
4 30	-0.2	-2.3	-2.3	-2.0	-0.7
5 00	+1.9	0	+4.9*	-1.7	-0.9
5 30	+3.2	+2.8	+2.6	-2.2	-0.4
6 00	+3.0	+3.4	+1.0	-1.7	-0.7
6 30	+1.9	+8.8*	-0.8	-1.9	-0.6
7 00	+0.7	Open sheet	-1.6	-1.9	-0.4
7 30	+0.9	—	-0.3	-1.4	-0.3
8 00	-0.5	-0.9	-0.9	-1.1	-0.4
8 30	-0.7	-0.5	+0.3	-1.2	-0.4
9 00	—	Sheet transferred to phosphate saline gassed with 100% O_2	+0.7	-0.7	-0.3†
9 15	—	—	—	-0.4†	+1.0
9 30	—	Q_{O_2}	—	+2.9	+1.0
9 45	—	-2.7	—	+4.9	+1.0
10 00	—	-2.4	—	+5.1	+0.9
10 15	—	-1.0	—	+4.7	—

* Perforation observed.

† Thiocyanate added from side arm (final concentration 0.02 M).

When HCl was secreted at rapid rates the secretory phase was followed by a period during which acid diffused out and liberated CO_2 from the bicarbonate in the medium (Table 3, *A* and *B*). Perforation of mucosa *B* resulted in increased evolution of CO_2 due to extrusion of some acid contents. In order to decide whether the evolution of CO_2 was due to diffusion or glycolysis, the following experiment was carried out. Two mucosae were removed from their cups, emptied, washed in saline and the open sheets replaced in bicarbonate saline, gassed with 5% CO_2 + 95% O_2 . Readings were continued, and a small uptake of gas showed that the previously observed evolution of CO_2 was not due to aerobic glycolysis. The tissues were then transferred to cups containing phosphate saline, gassed with 100% O_2 , and incubated for a further 1.5 hr. Their average Q_{O_2} during this period was -2.0 (see Table 3, *B*).

In one very large mucosa (Table 3, *A*) the rate of back diffusion of H^+ ions reached a peak between 5.5 and 6 hr, and then decreased. At 8 hr CO_2 evolution stopped and a small uptake of gas of the same magnitude, as with open tissue, showed that the tissue was still alive and now in the resting state. On removal from the cup this mucosa was inspected, the bag was only about half filled, undamaged, and showed no signs of perforation even on squeezing. It contained a clear watery fluid admixed with particles of opaque material, apparently derived from cell debris. Buffering by this cell material and the loss of H^+ ions by diffusion most probably account for the relatively high pH observed (2.9).

The molarity of the HCl solution in the secretion at the end of the secretory period (i.e. before back diffusion started) was calculated (a) from the amount of extra CO_2 taken up (equivalent to the amount of HCl produced (Davies, 1948)), which was measured manometrically, and the volume of the secretion (increase of the wet weight of the tied mucosa), and (b) by titration of the H^+ in the gastric contents recovered after incubation, and correction for the amount of H^+ lost by diffusion (amount of CO_2 evolved in the manometric experiment), and for the difference between the wet weights of contents and secretion.

It should be noted that the pH values given in Table 2 were determined after the completion of the experiments. They do not, therefore, represent the pH of the gastric contents during the experiments, although they give some indication of the relation between the pH and the rate of diffusion. Davies & Longmuir (1948) found that the pH of the contents of tied mucosa, incubated in bicarbonate saline for short periods, can be close to 1, the highest concentration of H^+ in gastric secretion reported by these authors was 0.109M. However, in experiments of longer duration, H^+ ion concentrations of less than 0.06M were observed by Davies (1948).

Inhibition of secretion. Addition of an inhibitor, thiocyanate, resulted in evolution of CO_2 due to back diffusion of H^+ ions, when the pH of the gastric contents was less than about 2. At the end of experiments on mucosae *D* and *E*, thiocyanate was added to a final concentration of 0.02M. The evolution of CO_2 which followed immediately was small in *E*, and was possibly due to aerobic glycolysis, since the pH of the contents, 3.48, was too high for diffusion to occur at a measurable rate, if at all. Mucosa *D*, however, showed a large evolution of CO_2 which was probably due to the combined effect of diffusion and glycolysis. The pH of its contents, measured after the experiment, was 1.84.

Diluting secretions. Since most workers emphasize the importance of dilution and neutralization of the secreted HCl by mucus (see Babkin, 1944; Hollander, 1938), attempts were made to estimate the neutralizing power of the mucus in frog stomachs. Eugenol, which has been shown to stimulate secretion of mucus in dogs (Hollander & Lauber, 1948), was used to increase the production of mucus by isolated frog gastric mucosa. Five tied mucosae were injected with 0.03–0.1 ml of an approximately 1% emulsion of eugenol in water (stabilized by 50 mg of cetyl alcohol containing ethylene oxide/100 ml), and placed in bicarbonate or phosphate saline in Warburg cups. After 1 hr incubation at 25°C, the contents of the bags were removed, weighed and titrated against 0.01N-HCl, in an atmosphere of 5% CO_2 + 95% O_2 . All the mucosae contained a viscous, opaque mucous material of pH 5.8–7.5. The titration curves showed that the samples were buffered between their initial pH and pH 3. In this pH range, their neutralizing capacity corresponded to that of 0.03–0.04N solutions of pure alkali.

Table 4. Diffusion coefficients of HCl

Tissue	$D \times 10^5$ (cm ² sec ⁻¹)
—	2.5*
Dead pig bladder	0.15
Anaerobic frog gastric mucosa	0.04
'Dead' frog gastric mucosa	0.02
Frog gastric mucosa (added HCl)	0.03
Frog gastric mucosa (secreted HCl)	0.01–0.016

* In aqueous solution at 19.3° (Thovert, 1902)

DISCUSSION

Evidence in support of the diffusion theory. Teorell's conclusions that the reduction of the acidity of the gastric juice is mainly due to the diffusion of H^+ ions through the gastric mucosa into the blood (Teorell, 1933) have been criticized by other workers (Hollander, 1938), particularly because of the conditions of the experiments, since the volume of the instilled HCl solutions had to be small in relation to the size

of the stomach in order that the change in acidity might be sufficiently large to be detected by analysis (Babkin, 1944). As a result, the diffusion theory does not appear to have found general acceptance.

In the experiments described in this paper relatively large volumes of HCl solution were placed in contact with the secretory sides of sheets of gastric mucosa, and the rate of diffusion of H^+ ions was followed by manometric measurement of the amount of CO_2 liberated from the bicarbonate solution on the nutrient side. With non secreting mucosa the rate of diffusion depended on the concentration of the HCl solution, and consequently decreased exponentially with time, in agreement with the diffusion theory (Teorell, 1947). The onset of secretion of HCl by the mucosa after histamine stimulation could reduce the output of CO_2 due to diffusion (Fig. 3), or result in a net uptake of CO_2 (Fig. 4). Since, under the conditions of the experiment, secretion of HCl is accompanied by an uptake of CO_2 equivalent to the amount of HCl produced (Davies, 1948), these results suggest that secretion and back diffusion can occur at the same time, and that the observed uptake or output of CO_2 is the balance of the amount of CO_2 taken up during secretion of HCl by the mucosa and the amount evolved by back diffusion of H^+ ions. This is supported also by the observation that the molarity of the HCl solution secreted by mucosae, whose secretory sides were in contact with HCl solutions, was low. If, however, on the assumption that secretion and back diffusion of H^+ ions can occur at the same time, the true Q_{HCl} was taken as the sum of the observed Q_{HCl} and the $q_{CO_2}^{H^+}$, then the calculated molarity was between 0.10 and 0.12N (Table 1). Thus, allowing for some dilution by mucus, is in agreement with the generally accepted view that the HCl solution secreted by the gastric glands is isotonic with the blood (Babkin, 1944).

The relation of back diffusion to secretion. In experiments in which HCl solutions were placed in contact with sheets of gastric mucosa, the diffusion rates were high ($D = 0.03 \times 10^{-5}$ cm²sec⁻¹ corresponds to a $q_{CO_2}^{H^+}$ of 12 with 0.1N HCl, which is of the order of the highest Q_{HCl} observed, and of 2.4 with 0.2N HCl, for a mucosa of 1.3 cm² secretory area and 8 mg dry weight). Although mucosae in contact with 0.1N HCl did not respond to histamine stimulation, it was at least possible to observe secretory responses when lower concentrations of HCl (0.05 and 0.02N) were used. Lower diffusion rates ($D = 0.01-0.015 \times 10^{-5}$ cm²sec⁻¹) were observed when 0.1N HCl was added to secreting tissue, or when the acid secreted by a tied mucosa was diffusing out at the end of the secretory phase. The reason for this difference is not clear, but it is possible that the tissue suffered some damage when HCl solutions were placed in contact with it before it had recovered from injury due to dissection and

manipulation. (For comparison of diffusion coefficients see Table 4.)

The relative contributions of dilution, neutralization and diffusion to the reduction of gastric acidity. If it is accepted that the primary acidity of the secreted HCl is 0.12N in the frog (isotonic with blood), an explanation is required for the low concentration of HCl in the secretion of tied mucosa at the end of the secretory phase. In the experiments in Table 2 the deficit of H^+ ions ranged from 43 to 56% (cf. Davies, 1948, Table 4). Possible explanations for the reduction of acidity are (a) dilution by a neutral secretion, (b) dilution and neutralization by a secretion of great neutralizing power, (c) loss of H^+ ions by back diffusion, and (d) a combination of (c) with (a) or (b).

In mucosa A the concentration of HCl in the secretion at the end of the secretory phase was 0.063N. This could correspond to a loss of 47% of the H^+ ions or to secretion of the corresponding amount of a neutral fluid. If it is assumed that back diffusion occurred while the HCl was being secreted, then the true rate of CO_2 uptake corresponding to the HCl produced was the sum of the observed Q_{HCl} and the $q_{CO_2}^{H^+}$. The value of the latter during active secretion could not be determined, but it can be assumed to be at least equal to the maximum $q_{CO_2}^{H^+}$ observed at a later stage. Thus the sum of the average Q_{HCl} (4.8) and the maximum $q_{CO_2}^{H^+}$ (3.2) would give the true average Q_{HCl} (8.0), and the primary acidity of the secreted HCl solution calculated from this and the average q_{H_2O} (3.4) would be 0.105N. Some reduction of the acidity may be due to neutralization by mucus. An estimate of the amount of mucus extruded from the mucous cells may be obtained from the difference between the wet weight of the contents and the secretion (cf. Davies, 1948), or from the difference between the wet weights of a mucosa before and after incubation, although this may include some cell debris. It seems reasonable to assume that only the mucus, present as such or as a precursor in the mucous cells at the beginning of the experiment, is extruded into the lumen, and that there is no regeneration of mucus by an isolated mucosa, incubated in an inorganic medium containing glucose as the only added substrate. The neutralizing capacity of gastric mucus in frogs was found to be equivalent to that of a solution of about 0.05N alkali (see p. 155). Since the difference between the initial and final wet weight (or between the wet weights of contents and secretion) of mucosa A was about 80 mg (Table 2), this amount, if equivalent to 0.05N alkali, could have neutralized about 90 μ l of the total 1600 μ l H^+ (590 mg secretion containing 0.12N HCl is equivalent to 1600 μ l H^+). Since the mucosa contained 830 μ l H^+ at the end of the secretory period of 4 hr, 680 μ l H^+ are left to be accounted for by diffusion. This amount corresponds to a $q_{CO_2}^{H^+}$ of 4.0, which is

greater than the maximum $q_{CO_2}^{H^+}$, observed. However, the neutralizing capacity of the mucus may have been greater than assumed in this calculation (but even if equivalent to 0.12N alkali, it could not have neutralized more than 200 μ l H⁺), and the rate of diffusion may have been higher during the period of active secretion than several hours later, when the H⁺ ion concentration was already reduced. It was also assumed that there was no appreciable amount of other diluting secretions. This seems to be justified on histological grounds, since in frog gastric mucosa, apart from the oxyphilic cells of the chief glands, all other cells of glands and surface epithelium are of mucoid character (Pernkopf & Lehner, 1937). It is therefore very probable that in the very active mucosa at the reduction of acidity during the secretory phase was mainly due to back diffusion, and that this was so to a large extent also in mucosae D and E, although with slow secretory rates, dilution and neutralization may be of relatively greater importance than with faster rates.

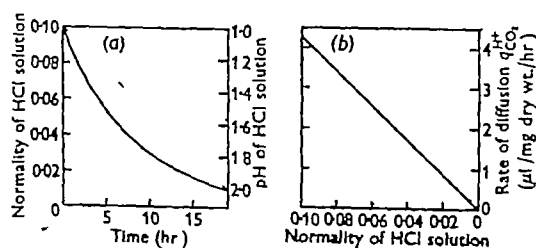


Fig 5 Diffusion of H⁺ ions through non secreting gastric mucosa. Data calculated for frog gastric mucosa, area 1 cm², thickness 0.3 mm, dry weight 6.0 mg, in contact with 0.1 ml. pure unbuffered HCl solution, $c = 2 \times 10^{-4}$ ml. min⁻¹, $D = 0.01 \times 10^{-5}$ cm² sec⁻¹. (a), unbroken line, decrease of normality of HCl with time, broken line, increase of pH with time, (b), relation of rate of diffusion to normality of HCl solution

The relation between rate of back diffusion and H⁺ ion concentration of the gastric contents Since the concentration of an HCl solution, in contact with a membrane through which it can diffuse, decreases exponentially with time, the rate of diffusion ($q_{CO_2}^{H^+}$) depends on the prevailing H⁺ ion concentration (Teorell, 1933), of which it is a linear function. The H⁺ ion concentration must be high (pH less than about 2) for diffusion to be fast enough to be measurable (see Fig 5, which has been constructed for an ideal non secreting mucosa in contact with pure, unbuffered HCl solution). This may explain why back diffusion was observed in highly active mucosa, but not in mucosa of low secretory activity. With fast rates of secretion, high concentrations of H⁺ ions are built up rapidly so that, when the rate of secretion decreases towards the end of the secretory phase, it is outstripped by the rate of back diffusion.

With low secretory rates the decrease in the H⁺ ion concentration due to dilution, buffering by mucus and cell material and to diffusion results in a reduction of the rate of back diffusion, so that it cannot overtake the rate of secretion which can be maintained at a low, but steady level for many hours. Since the observed CO₂ uptake is the resultant of the CO₂ uptake due to HCl secretion and the CO₂ output due to back diffusion, inhibition of secretion should result in the evolution of CO₂, due to diffusion only, provided the H⁺ ion concentration of the gastric contents is sufficiently high (Table 2, D).

The experiments described in this paper show that H⁺ ions can diffuse through living isolated gastric mucosa and support Teorell's diffusion theory (Teorell, 1933, 1947). They suggest that back diffusion of H⁺ ions also occurs during secretion of HCl, thus reducing the efficiency of the process. In the frog, the loss of H⁺ ions by back diffusion during the secretory phase can be relatively large, in the mammal, however, owing to the greater thickness of the mucosa and the high secretory rates (ten times as fast as in the frog) the loss of H⁺ ions may be comparatively small.

SUMMARY

1 The diffusion of H⁺ ions through isolated gastric mucosa of frogs and toads was studied by gasometric methods.

2 Solutions of HCl were placed in contact with the secretory side of sheets of gastric mucosa, which were incubated in bicarbonate saline. With non secreting tissue an output of CO₂ was observed which decreased exponentially with time, in agreement with the findings of Teorell (1933, 1947).

3 On stimulation of mucosa in contact with HCl by 5×10^{-5} M histamine, the evolution of CO₂ ceased, and the subsequent rates of CO₂ uptake and fluid secretion corresponded to the production of HCl solutions of low concentration. If, however, it was assumed that the rate of CO₂ uptake had been reduced by simultaneous evolution of CO₂ due to back diffusion of H⁺ ions, the calculated concentration of the secreted HCl solution was near isotonicity.

4 In prolonged experiments with highly active, tied gastric mucosa incubated in bicarbonate saline, back diffusion of H⁺ ions was observed at the end of the secretory phase. Mucosa of low secretory activity, however, continued to take up CO₂ for at least 10 hr. Inhibition of secretion by 0.02M thiocyanate was followed by back diffusion when the pH of the gastric contents was less than 2.

5 At the end of the secretory phase the concentration of HCl in the secretion was 40–60% of the theoretical 0.12N. The loss can be largely accounted for by back diffusion of H⁺ ions during the secretion of HCl.

6 The results show that back diffusion of H^+ ions can occur in living isolated frog gastric mucosa, and that the rate of diffusion is linearly related to the H^+ ion concentration of the gastric contents. They suggest that the concentration of HCl in the secretion is reduced by back diffusion not only after the

cessation of secretory activity, but also during the period of HCl production

The author wishes to thank Prof. H. A. Krebs, F.R.S., for help and encouragement, and Dr R. E. Davies for suggesting this investigation and for criticism and advice

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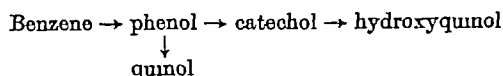
Studies in Detoxication

26 THE FATES OF PHENOL, PHENYLSULPHURIC ACID AND PHENYLGLUCURONIDE IN THE RABBIT, IN RELATION TO THE METABOLISM OF BENZENE

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(Received 11 March 1949)

From studies of the fate of benzene (Porteous & Williams, 1949*a, b*), catechol (Garton & Williams, 1948), resorcinol and quinol (Garton & Williams, 1949) in the rabbit, the following scheme for the oxidation of benzene was suggested



In order to obtain further evidence about this scheme and to find out whether phenol conjugates were oxidized, the metabolic fates of phenol, phenylsulphuric acid and phenylglucuronide were studied in detail.

Porteous & Williams (1949*a*) showed that when phenol is fed orally to rabbits practically all the phenol excreted is conjugated. The conjugated phenol excreted accounted for 80–90% of the phenol fed, although the total conjugation (i.e. glucuronic acid and ethereal sulphate) accounted for 100%. The difference between the total conjugation and conjugated phenol, amounting to some 10%, was assumed to be oxidation products of phenol. The nature of these oxidation products is described here.

Quinol is known, by isolation, to be an oxidation product of phenol in the dog (Baumann & Preusse, 1879*a, b*). Brieger (1879) claims to have isolated catechol and quinol from 40 l of urine obtained from hospital patients treated with phenol, but no reference is made to the quantities isolated or to the amount of phenol administered.

Phenylsulphuric acid is well known to be a metabolite of phenol. On feeding the compound to rabbits, 80–95% can be accounted for in the urine as ethereal sulphate (Rhode, 1923, see also Auerbach, 1879 and Christiani, 1878). Baumann & Preusse (1879*b*) reported that after feeding the potassium salt to a dog, quinol but not catechol could be detected in the hydrolysed urine. Furthermore, Sperber (1948*b*) has shown that, on injection of the sodium or potassium salts of phenylsulphuric acid into hens, some 75% can be recovered in the urine in 80 min and there is no rise in the glucuronide output. These results indicate that phenylsulphuric acid is rapidly eliminated from the body mainly as such, but a small proportion may be oxidized to quinol.

The other well known metabolite of phenol is phenylglucuronide. Nakano (1937) found that this compound, on injection, is excreted almost quantitatively.

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tively unchanged, but, when fed, only about a half is excreted in the urine and there is a rise in the ethereal sulphate output. Sperber (1948b), however, found that when injected into hens 57 % of the dose could be recovered in the urine as glucuronide. There are no reports of phenylglucuronide giving rise to oxidation products.

The study of these two conjugates was undertaken because we wanted to find out whether free phenol or conjugated phenol was the precursor of the polyphenols of benzene urine.

EXPERIMENTAL AND RESULTS

I Excretion of ethereal sulphate and of glucuronide after administration of phenylsulphate or phenylglucuronide

Glucuronic acid was determined colorimetrically (Hanson, Mills & Williams, 1944) and ethereal sulphate gravimetrically by Fohn's method (cf. Williams, 1938).

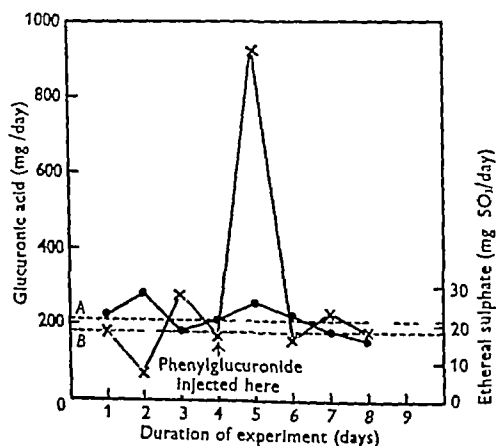


Fig 1 The effect of injecting phenylglucuronide (1.21 g) on the ethereal sulphate and glucuronic acid outputs of rabbit no 130, ●—●, ethereal sulphate, x—x, glucuronic acid. The dotted lines show the average normal output, A of ethereal sulphate and B of glucuronic acid.

The potassium phenylsulphate used was prepared according to Burkhardt & Lapworth (1926) and phenyl glucuronide dihydrate according to Porteous & Williams (1949a) and Garton, Robinson & Williams (1949). The constants for these compounds are as recorded by Garton *et al.* (1949).

The rabbits used were kept on a diet of Lever cubes (50 g/day) and water *ad lib* during experimental periods. This diet appeared to cause the least fluctuations in the normal ethereal sulphate and glucuronic acid outputs.

For feeding experiments potassium phenylsulphate and phenylglucuronide dihydrate were dissolved in water. For the injection experiments 2.42 g of phenylglucuronide dihydrate were dissolved in 40 ml water. This solution was maintained at 30° and 10 ml injected into the loose skin of the groin at each side, each animal thus receiving 20 ml (i.e. 1.21 g of the glucuronide).

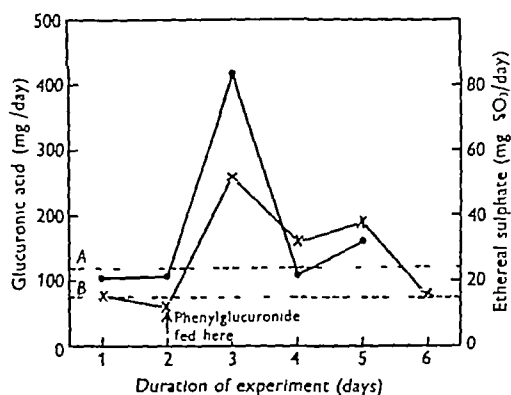


Fig 2 The effect of oral administration of phenylglucuronide (1.5 g) on the ethereal sulphate and glucuronic acid outputs of rabbit no 124, ●—●, ethereal sulphate, x—x, glucuronic acid. The dotted lines show the average normal output, A of ethereal sulphate, B of glucuronic acid.

The results are recorded in Table 1, which shows that when potassium phenylsulphate is fed some 90 % of it can be accounted for as 'extra' ethereal sulphate. The glucuronic acid output does not appear to be

Table 1 The excretion of ethereal sulphate and glucuronic acid by rabbits receiving potassium phenylsulphate or phenylglucuronide

Compound and route of administration	Rabbit no	Dose		'Extra' ethereal sulphate (% of dose)	'Extra' glucuronic acid (% of dose)	Total recovered (% of dose)
		g	mg/kg			
Potassium phenylsulphate						
Oral	124	0.33	100	82.9	0	82.9
Oral	125	0.36	100	90.3	0	90.3
Oral	126	0.36	100	89.0	0	89.0
Oral	124	1.65	500	—	0	—
Oral	125	1.80	500	—	0	—
Phenylglucuronide						
Oral						
Oral	124	1.40	500	21.3	28.4	49.7
Oral	125	1.40	500	15.4	41.5	56.9
Subcutaneous injection	129	1.21	403	0	98.5	98.5
Subcutaneous injection	130	1.21	378	0	96.2	96.2

disturbed. At a dose level of 100 mg ($\equiv 44$ mg phenol)/kg body weight a small rise in glucuronic acid cannot be readily detected by the quantitative method used. Two determinations of glucuronic acid output were therefore made at the higher level of 500 mg ($\equiv 222$ mg phenol)/kg, but again no disturbance of the normal glucuronic acid level could be detected. It is concluded, therefore, that very little potassium phenylsulphate is hydrolysed in the intestine or in the body. If hydrolysis had been extensive then the excretion of phenylglucuronide would have been expected to rise.

Table 1 shows clearly that the fate of phenylglucuronide depends on the route of administration. On injection the glucuronide is almost quantitatively recovered in the urine in 1 day (see Fig. 1). On oral administration, however, there is an appreciable rise in the ethereal sulphate output and only about 50% of the glucuronide fed can be accounted for (i.e. by the 'extra' ethereal sulphate and glucuronic acid). The excretion of 'extra' glucuronic acid continues for 3 days after feeding, although the excretion of 'extra' sulphate is complete in 1 day (see Fig. 2). That there is a prolonged excretion of glucuronide after feeding phenylglucuronide is supported by qualitative findings reported below.

II Isolation of the oxidation products of phenol

(1) *Preparation of extracts* Twelve rabbits were each given twice a day on two successive days 0.25 g phenol in 10 ml water by stomach tube (total fed, 12 g). On the first day 1975 ml. urine were collected and on the second 2000 ml. The slightly alkaline urine was non-reducing and a little darker than normal. It gave an intense Tollens test, but no FeCl_3 test though small amounts of free phenol were detected with 2,6-dichloroquinone chloroimide. No other free phenols were detected. Each day's urine was filtered, made normal with respect to HCl and then heated for 0.5 hr at 94° on a water bath to hydrolyse ethereal sulphates (cf. Garton *et al.* 1949). The urines were then extracted to exhaustion (30 hr) with peroxide-free ether. Two extracts, A_E (for day 1) and B_E (for day 2) were thus obtained.

The extracted urines were now made 3N with respect to HCl, boiled under reflux for 2 hr to hydrolyse glucuronides and again extracted with ether for 30 hr. Two further extracts A_G and B_G were thus obtained.

The four extracts A_E , B_E , A_G and B_G were evaporated at 20° to about 20 ml and examined for phenols by colour tests as described by Porteous & Williams (1949b). A_E and B_E were found to contain phenol, catechol, quinol and hydroxyquinol. A_G and B_G gave strong tests for phenol and very weak tests for catechol. None of the extracts contained resorcinol, pyrogallol or phloroglucinol. It was clear from these tests that the oxidation products of phenol were almost entirely in the ethereal sulphate fraction.

(2) *Isolation of phenols from the ethereal sulphate fraction* Extracts A_E and B_E were combined, 30 ml. water added and the ether removed at 20° . The yellow-brown aqueous residue was boiled with charcoal and filtered. The cooled filtrate was made just alkaline with NaHCO_3 and treated with saturated

basic lead acetate until precipitation was complete. The filtrate was freed of Pb with H_2S and extracted with ether for 20 hr. Evaporation of the ether at 20° in the presence of 10 ml. water gave an aqueous residue which was then treated with 50 ml. saturated NaHCO_3 and 20 ml. acetone containing 1 g *p*-toluenesulphonyl chloride. After shaking the mixture for an hour and then keeping overnight, the whole was diluted with 150 ml. water. A crystalline product (980 mg, m.p. $80-90^\circ$) was obtained, which on fractional crystallization from absolute ethanol (charcoal) yielded (in order of crystallization) (a) quinol di-*p*-toluenesulphonate (50 mg, m.p. 155°), on recrystallization it had m.p. 155° and mixed m.p. 158° with authentic material, but depressed the m.p. of catechol di-*p*-toluenesulphonate, (b) catechol di-*p*-toluenesulphonate (15 mg), m.p. 160° and mixed m.p. 160° , it depressed the m.p. of the quinol ester, (c) phenol *p*-toluenesulphonate (675 mg), m.p. and mixed m.p. $91-92^\circ$. We were unable to isolate the hydroxyquinol derivative, although colour reactions indicated very definitely that hydroxyquinol was present in the ethereal sulphate fraction.

The basic lead acetate precipitate was suspended in water and the lead removed with H_2S . The lead-free filtrate was treated in the same way as above (yield of toluenesulphonates, 2.27 g) resulting in the isolation of quinol di-*p*-toluenesulphonate (136 mg), m.p. and mixed m.p. $158-159^\circ$, and phenol *p*-toluenesulphonate (2 g), m.p. and mixed m.p. 92° , each after recrystallization.

The yields of the phenols are summarized in Table 2.

Table 2 Phenols isolated from the ethereal sulphate fraction of the urine of rabbits receiving phenol orally

Compound isolated	Yield of <i>p</i> -toluene sulphonate ester (g)	Yield as percentage of dose of phenol
Phenol	2.67	8.5
Quinol	0.186	0.41
Catechol	0.015	0.033
Hydroxyquinol	—	Small amounts detected by colour reactions

(3) *Isolation of phenol from the glucuronide fraction* The only hydroxybenzene which appeared to be present in quantity in extracts A_G and B_G was phenol itself. Traces of catechol were detected by colour reactions. Treatment of the combined ether-free fractions A_G and B_G with *p*-toluenesulphonyl chloride in acetone yielded 3.15 g of phenol *p*-toluenesulphonate (m.p. and mixed m.p. 92° after recrystallization) corresponding to 10% of the dose. This low yield of phenol indicates either that the glucuronide fraction was incompletely hydrolysed or that phenol is lost in the more vigorous hydrolysis by combination with other urinary constituents. It was therefore decided to investigate the glucuronide fraction obtained directly from phenol urine by systematic lead acetate precipitation.

(4) *Examination of the basic lead acetate fraction of phenol urine* Phenol (0.8 g) was fed to each of six rabbits and an 18 hr. urine (750 ml) collected. The basic lead acetate precipitate was prepared as for the preparation of phenylglucuronide (Porteous & Williams, 1949a) and the Pb removed by H_2S . From the Pb-free filtrate 4.23 g of phenylglucuronide dihydrate were isolated. This corresponds to 31% of the dose. The concentrated mother liquors (16 ml) from the glucuronide were now examined for polyphenols.

They were hydrolysed by refluxing for 1 hr with 6 ml conc H_2SO_4 . The hydrolysate was cooled and diluted with water to 50 ml and then extracted with peroxide free ether for 8.5 hr. The extract thus obtained was tested for phenols, and it was found that only phenol itself was present, although doubtful tests for quinol were obtained. It was therefore concluded that only phenol occurs in the glucuronide fraction of phenol urines. The traces of catechol and quinol were insignificant.

Urines were collected for a further 3 days after the urines examined above. These urines appeared to be normal. They were, however, subjected to the same procedures as described above for the urines of the first 2 days. The extracts obtained did not contain significant amounts of phenolic metabolites and we detected traces of phenol and catechol in the ethereal sulphate fractions only. These traces were no more than can be found in similarly treated extracts of normal rabbit urine.

III The metabolites of potassium phenylsulphate

(1) *Examination of the ethereal sulphate fraction of phenyl sulphuric acid urine*. Ten rabbits were each given 2 g of

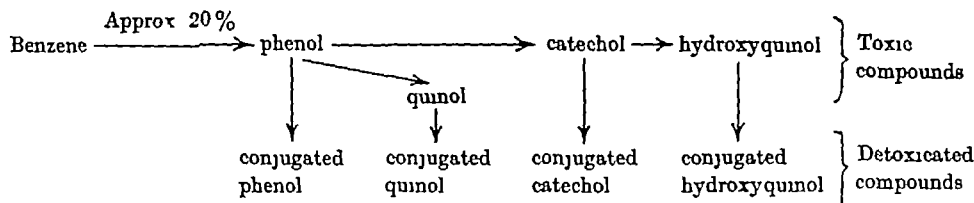
sodium phenylsulphate does not lead to an increased excretion of phenolic glucuronides.

IV The metabolites of phenylglucuronide

Two rabbits were each given by stomach tube an aqueous solution containing 1.5 g of phenylglucuronide dihydrate. Attempts to isolate the phenols of the ethereal sulphate and glucuronide fractions were not made in this case. We were, however, able to detect, besides phenol, definite but small amounts of catechol, quinol and hydroxyquinol in the ethereal sulphate fraction. In the glucuronide fraction the only hydroxybenzene present was phenol.

DISCUSSION

On the basis of the results of the present paper and of those of Porteous & Williams (1949a, b) and Garton & Williams (1948, 1949), the following scheme for the metabolism of benzene to hydroxyquinol is suggested.



potassium phenylsulphate in water by stomach tube and a 24 hr urine (950 ml.) collected, and filtered through glass wool. The non-reducing urine was made normal with respect to HCl and hydrolysed by warming to 94° for 30 min. The liberated phenols were then extracted (20 hr) with peroxide-free ether. After adding 100 ml water, the ether was removed *in vacuo* at 20°. The residual liquor, which contained globules of dark red oil, was boiled with charcoal and filtered. The filtrate contained much phenol, small amounts of quinol and only slight traces of catechol and hydroxyquinol.

Since the amounts of quinol present were small, it was decided to estimate it on a sample of the solution before attempting isolation. The quinol was estimated iodometrically as described in an earlier paper (Garton & Williams, 1949) by the method of Wieland (1910). It was found that 31 mg of quinol were present or 0.3% of the potassium phenylsulphate fed.

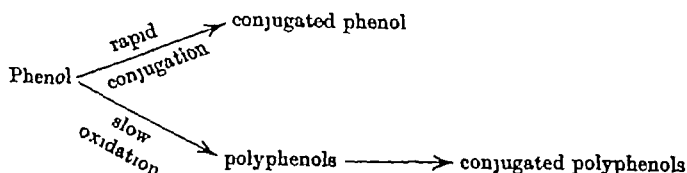
We attempted to isolate the quinol as a *p*-toluenesulphonate ester, but without success. Our main product was phenol *p*-toluenesulphonate, m.p. 92° (12.7 g. or 54% of the dose).

(2) *The glucuronide fraction of phenylsulphuric acid urine*. The above partially hydrolysed urine was warmed to expel ether and then made 10N with respect to H_2SO_4 . The mixture was refluxed for 1 hr and subjected to continuous ether extraction. Only slight traces of phenol were found. This finding confirms the view that the administration of potas-

sium phenylsulphate in water by stomach tube and a 24 hr urine (950 ml.) collected, and filtered through glass wool. The non-reducing urine was made normal with respect to HCl and hydrolysed by warming to 94° for 30 min. The liberated phenols were then extracted (20 hr) with peroxide-free ether. After adding 100 ml water, the ether was removed *in vacuo* at 20°. The residual liquor, which contained globules of dark red oil, was boiled with charcoal and filtered. The filtrate contained much phenol, small amounts of quinol and only slight traces of catechol and hydroxyquinol.

According to the above scheme it is free phenol and not conjugated phenol which gives rise to polyphenols. The evidence for this view will occupy the rest of this discussion.

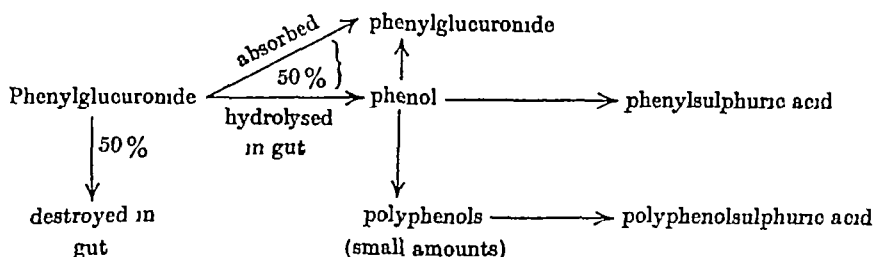
Porteous & Williams (1949a) have shown that about 21% of the benzene administered orally to rabbits is oxidized to phenols and the ratio phenol/polyphenols of the urine is roughly 1, i.e. phenol and the total polyphenols (catechol, quinol and hydroxyquinol) are produced in roughly equal amounts. On administration of phenol, however, the ratio phenol/polyphenols is about 5 or 6. This means that administered phenol is rapidly conjugated forming phenylsulphuric acid and phenylglucuronide which are (see p. 162) excreted unchanged. This rapid conjugation leaves only a small proportion of free phenol for further oxidation, unless there is a reversal of conjugation, thus



That the glucuronic acid and ethereal sulphate conjugations are rapid processes is now fairly well established (see Hemingway, Pryde & Williams, 1934, Sperber, 1948*a*)

Earlier workers have shown that the potassium or sodium salts of phenylsulphuric acid whether fed by mouth or injected are rapidly excreted unchanged almost quantitatively (Christiani, 1878, Rhode, 1923, Sperber, 1948*b*) Our results confirm this view for we recovered 90 % of orally administered potassium phenylsulphate within 24 hr of feeding. If this compound had undergone hydrolysis in the body to any significant extent, then a rise in the glucuronic acid output would have been noted. No such rise

there is a sharp rise in ethereal sulphate corresponding to 15–20 % of the dose of glucuronide. Furthermore, in the ethereal sulphate fraction of the urine, phenol, quinol, catechol and hydroxyquinol are detectable. The excretion of glucuronic acid remains above normal for 3 days after dosing (see Fig 2), and this probably means that the glucuronide is poorly absorbed from the intestine. Poor absorption of the glucuronide from the intestine would thus allow considerable hydrolysis and destruction by intestinal bacteria (Barber, Brooksbank & Haslewood (1948) have shown that certain bacteria destroy glucuronides). The fate of phenylglucuronide on oral administration can be represented as follows

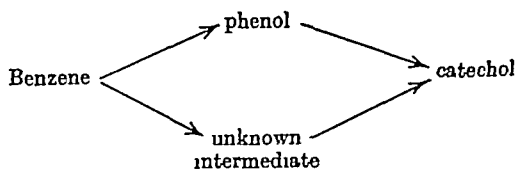


was observed even when the dose of the ethereal sulphate was as high as 500 mg/kg and the conclusion was confirmed by our inability to find phenylglucuronide when the glucuronide fraction of the urine was examined qualitatively. We did find, however, in the ethereal sulphate fraction of 'potassium phenylsulphate urine' small amounts of quinol (estimated as 0.3 % of the dose) and extremely small traces of catechol and hydroxyquinol. These could have arisen from a small amount of free phenol released by a slight hydrolysis of phenylsulphuric acid by the phenolsulphatase of the gut. However, the possibility of direct oxidation of phenylsulphuric acid to quinolmonosulphuric acid cannot be completely ruled out, although we feel that this is unlikely because a strong acid such as phenylsulphuric acid, once in the body, would be too rapidly excreted to allow much oxidation. Both phenylsulphuric acid and phenylglucuronide are eliminated by renal tubular excretion in hens (Sperber, 1948*b*).

On injection, phenylglucuronide is excreted quantitatively unchanged in 24 hr thus confirming the work of Nakano (1937) on rabbits and of Sperber (1948*b*) on hens. If any hydrolysis had taken place then a rise in ethereal sulphate would have occurred (see Fig 1). It is clear that glucuronidase does not exert its hydrolytic function in the intact animal under the conditions of our experiments.

On oral administration, however, phenylglucuronide undergoes considerable hydrolysis and probably destruction. Only about 50 % of the dose could be accounted for in the urine, thus confirming the work of Nakano (1937). On the first day after feeding

There is one further point which requires discussion and that is the conversion of phenol to catechol. The semi quantitative results of Porteous & Williams (1949*a, b*) show that catechol and quinol are produced during the metabolism of benzene in equal amounts, whereas with phenol ten times as much quinol is produced as catechol. Quinol is thus the main oxidation product of phenol. This could mean that catechol is produced from benzene in more than one way, thus



Such an intermediate could be 1,2-dihydro-1,2-dihydroxybenzene which could yield both phenol and catechol as suggested by Porteous & Williams (1949*b*).

SUMMARY

1 The metabolic fates of phenol, phenylsulphuric acid and phenylglucuronide have been studied in the rabbit.

2 Besides forming the main metabolites, phenylsulphuric acid and phenylglucuronide, phenol is oxidized to a small extent to quinol, catechol and hydroxyquinol, which occur only in the ethereal sulphate fraction of the urine. The main oxidation product is quinol. Both quinol and catechol were isolated as *p*-toluenesulphonates from phenol urine.

3 Orally administered potassium phenylsulphate and injected phenylglucuronide are excreted almost quantitatively unchanged by the rabbit

4 Orally administered phenylglucuronide causes a considerable rise in etheral sulphate output and small amounts of quinol, catechol and hydroxy-quinol are also excreted. The evidence suggests that phenylglucuronide is partly hydrolysed in the gut

yielding free phenol which is then oxidized to poly-phenols

5 The results are discussed and the conclusion is reached that the polyphenols of benzene and phenol metabolism are products of the oxidation of free phenol and not of conjugated phenol

The expenses of this work were in part defrayed by a grant from the Medical Research Council

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The Biogenesis of Porphyrins

THE DISTRIBUTION OF ^{15}N IN THE RING SYSTEM*

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(Received 24 January 1949)

It was shown by Shemin & Rittenberg (1946a, b) that feeding of glycine labelled with ^{15}N to men or rats produces a relatively high isotope content in the porphyrin moiety of the haemoglobin molecule. The effect appeared to be specific, since ammonia, glutamic acid, leucine and proline, also labelled with the stable nitrogen isotope, were relatively ineffective in this respect. These experiments did not indicate whether the isotopic nitrogen was equally distributed over the four rings of protoporphyrin, and this paper describes in the first place experiments designed to investigate this question. Degradation of porphyrins which is required for this purpose can be done by either an oxidative or a reductive procedure. The latter gives rise to alkylated pyrroles which are fairly unstable to light and air. After some preliminary experiments, in which reduction with sodium alkoxides was used, this method was abandoned. The method finally chosen consisted of reduction

of protoporphyrin to mesoporphyrin, followed by oxidation of the latter with chromic anhydride. The main products of this oxidation are methylethyl maleic acid imide and 1 methyl 2,2' carboxyethyl-maleic acid imide (haematonic acid imide)†. The neutral compound is derived from the rings I and II and the acid is produced from the two acidic rings III and IV. The chromic anhydride oxidation has been used extensively in the porphyrin field and has been examined systematically by Fischer & Wenderoth (1939). However, their method could not be successfully applied to the small quantities of porphyrins available to us, and it was necessary therefore to study the conditions of oxidation of mesoporphyrin and the separation of the products in the pure state in some detail. An investigation of

† In the German literature the term haematonic acid is used both for the above and for the corresponding diketo dihydrofuran derivative, we propose to describe the former as haematonic acid imide and the latter as haematonic acid anhydride.

* Preliminary communication, see Muir and Neuberger, 1948

the ultraviolet absorption spectra, dissociation constants and solubilities in various solvents of the various maleic acid derivatives was therefore undertaken, and on this basis a method of oxidation and separation elaborated which permitted the isolation of the two imides in crystalline form on a scale of about 10–15 mg. A convenient synthesis of the two imides is also described in the Experimental section.

By using glycine labelled with ^{14}C on the carboxyl carbon Grinstein, Kamen & Moore (1948) showed that the carboxyl group of glycine does not appear in the protoporphyrin molecule. It thus appeared possible that the immediate precursor of part of the haemin molecule is not glycine, but another nitrogenous compound derived from it. One possible pathway appeared in the conversion of glycine to serine which has been shown to occur *in vitro* (Winnick, Friedberg & Greenberg, 1948) and the decarboxylation of the latter to ethanolamine. This hypothesis was tested by feeding ethanolamine, labelled with ^{15}N .

EXPERIMENTAL

Preparation of methylethylmaleic acid imide and of haematinic acid imide

Methylethylmaleic anhydride and haematinic acid anhydride have been prepared by addition of hydrogen cyanide in a heterogeneous medium to the corresponding substituted acetoacetates, followed by hydrolysis and dehydration (Michael & Tissot, 1892, Kuster, 1906, 1908, 1924, Kuster & Weller, 1917). We have found that the yields are improved and the preparation of the cyanohydrin simplified if the condensation of the acetoacetate is carried out with liquid hydrogen cyanide under the catalytic influence of a small amount of potassium cyanide (Linstead & Millidge, 1936). Küster (1908, 1924) prepared the imides from the anhydrides by treatment with ammonia, yields by this method are not good and separation from other products is rather laborious. It was found that the imides can conveniently be prepared by heating the anhydrides with urea (see Cohen, 1928). This method, which gives very good yields with disubstituted maleic acid anhydrides, cannot be used for maleic anhydride itself and gives only poor yields with citraconic anhydride.

Methylethylmaleic acid anhydride. Ethyl 1 ethylacetoacetate (174 g, b.p. $97^{\circ}/18\text{ mm}$) was added slowly at -5° to liquid HCN (51 g) containing saturated aqueous KCN (1 ml). The mixture was left at 0° for 18 hr. and then at 20° for 2 hr., it was then poured into 36% (w/v) HCl (700 ml) previously cooled to 0° . The solution was left for 3 days at 0° , and after removal of excess HCN by aeration was heated first to 50° for 3 hr. and then to 100° for 12 hr. On cooling NH_4Cl crystallized out and was removed by filtration. The solution was evaporated to dryness *in vacuo* and the residue extracted

with dry boiling ethyl acetate (800 ml). After drying and removal of the solvent, the residue was distilled at $210\text{--}220^{\circ}$ (80 mm). The mixture of anhydride and water was separated after addition of NaCl. The anhydride was redistilled first at atmospheric and then under reduced pressure, boiling point was $233\text{--}234^{\circ}/760\text{ mm}$ and $121\text{--}123^{\circ}/18\text{ mm}$. Yield was 55% of the theoretical.

Methylethylmaleic acid imide. The above anhydride (14 g) and urea (6.5 g) were heated together at 160° in a flask fitted with an air condenser until the evolution of NH_3 ceased (40 min). The solid, including the copious sublimate, was taken up in a small amount of boiling water and allowed to cool. The crystalline material was filtered off and recrystallized from water, it had m.p. 68° which did not change on further recrystallization (Found N, 9.9, calc. for $\text{C}_7\text{H}_9\text{O}_4\text{N}$ N, 10.1%). Yield was 70% of the theoretical.

Haematinic acid anhydride. Ethyl 1 acetylglutarate (185 g, Clemons & Welch, 1928) was added slowly at -5° to liquid HCN (26 g) containing 0.7 ml of saturated aqueous KCN. The mixture was left at 0° for 18 hr. and at 20° for 2–3 hr. Hydrolysis of the cyanohydrin was carried out as described above. The solution, after being heated at 100° for 12 hr., was evaporated *in vacuo* to dryness and the residue extracted repeatedly with dry boiling ethyl acetate (15 l). The combined extracts were dried and the solvent removed *in vacuo*. The residue was dissolved in water (600 ml.) and extracted thrice with CHCl_3 . The aqueous solution was again evaporated to dryness and the residue taken up in ethyl acetate. The solvent was removed *in vacuo* until the volume of the solution was about 300 ml., 50 ml. portions were now heated in a flask immersed in a bath which was slowly heated to 185° . A second thermometer was immersed in the liquid. Whilst the dehydration proceeded the inside temperature was considerably lower than the outside. At the end of the reaction this difference became small ($5\text{--}10^{\circ}$). If the heating was prolonged beyond this point decarboxylation occurred. The solid which formed on cooling was dissolved in hot water (40 ml) and extracted with CHCl_3 (300 ml). The CHCl_3 extracts yielded after drying and evaporation *in vacuo* a crystalline residue which was recrystallized from ether and light petroleum ($60\text{--}80^{\circ}$). Melting point was 99° (uncorr.). Yield was 65%.

Haematinic acid imide. A mixture of the haematinic acid anhydride (3.65 g) and urea (1.5 g) was heated at $150\text{--}155^{\circ}$ for 40 min. The residue was dissolved in water (10 ml) and 36% (w/v) HCl (4 ml.) was added. The solution was extracted with ethyl acetate. The crystalline material which was left after removal of the solvent was recrystallized from ether and light petroleum (b.p. $60\text{--}80^{\circ}$). The melting point was 110° and the yield 75%. On recrystallizing the material twice from a little water the melting point rose to 117° (uncorr.) (Found N, 7.7, calc. for $\text{C}_8\text{H}_9\text{O}_4\text{N}$ N, 7.6%).

The methyl ester of the imide was obtained as follows. 0.5 g of the imide acid was dissolved in cold anhydrous methanol (20 ml.) containing HCl (1 N). The solution was left at 0° for 40 hr. and then concentrated *in vacuo*. The residue was dissolved in ether (40 ml) and extracted twice with NaHCO_3 solution and once with saturated NaCl (3 ml). The ether solution was dried and the solvent removed. The residue was distilled at 5 mm pressure at 160° . The distillate, which soon crystallized, was dissolved in CHCl_3 and precipitated by light petroleum (b.p. $40\text{--}60^{\circ}$). Melting point was 65° (uncorr.), Küster (1908) gives m.p. 64° (Found N, 7.0, calc. for $\text{C}_8\text{H}_{11}\text{O}_4\text{N}$ N, 7.1%).

Properties of the maleic acid derivatives

Ultraviolet absorption spectra A Beckman photo-electric spectrophotometer was used. The results are shown in Figs 1, 2 and 3. Both imides have a maximum in water, which is not very sharp, at 293–5 $m\mu$, this is shifted in ether to about 275 $m\mu$. Methyleneethylmaleic anhydride is very insoluble in water, and its absorption was therefore measured in ether. The two anhydrides have no maximum above 260 $m\mu$, and their absorption at 310 $m\mu$ is very small, i.e. only about 10% of that of the corresponding imides. Measurement of ultraviolet absorption at 275, 295 and 310 $m\mu$ can thus be

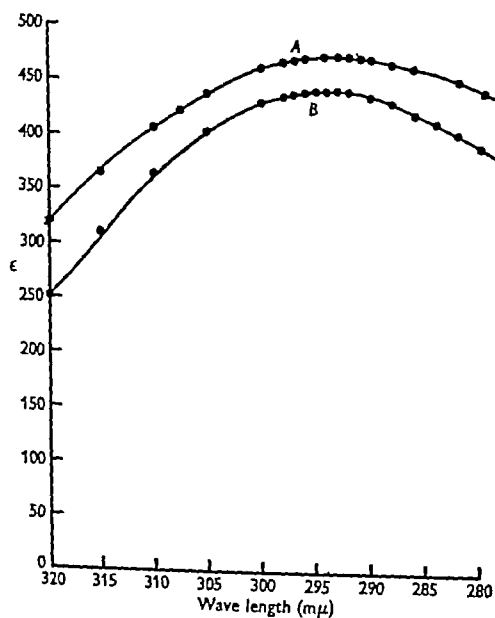


Fig 1 Absorption spectrum of haematinic acid imide (A) and methylethylmaleic acid imide (B) in water. ϵ in Figs 1, 2 and 3 denotes molecular extinction coefficient

used to determine approximately the relative concentrations of imides and anhydrides in a mixture, provided that other substances absorbing in this region are absent. The relatively intense absorption of the four disubstituted maleic acid derivatives in the region 250–300 $m\mu$ is in marked contrast to the weak absorption of maleic anhydride itself (Friedl, 1924).

The ultraviolet absorptions of the two haematinic acids in ether have been measured by Friedl (1924).

Dissociation constants The apparent dissociation constants of the two haematinic acids were determined by titrating 20 ml portions of 0.01N solutions with 0.1N sodium hydroxide at $22 \pm 1^\circ$ using a Murhead pH meter calibrated in the usual manner. The results are shown in Fig 4. Haematinic acid

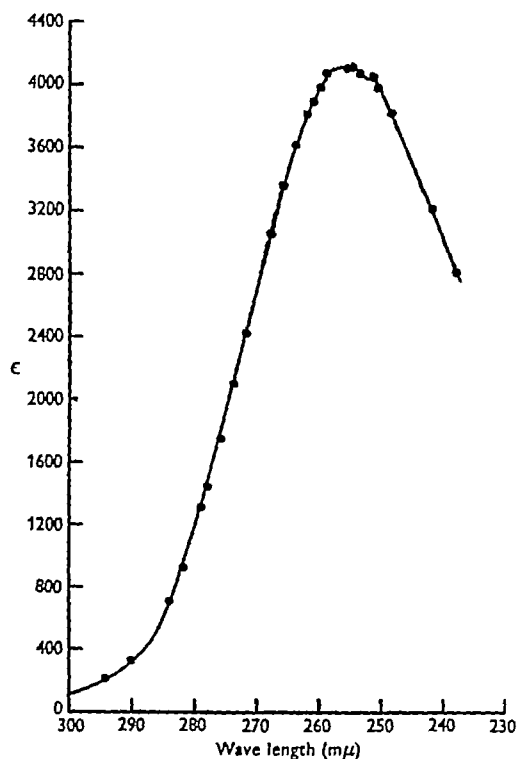


Fig 2 Absorption spectrum of methylethylmaleic acid anhydride in ether

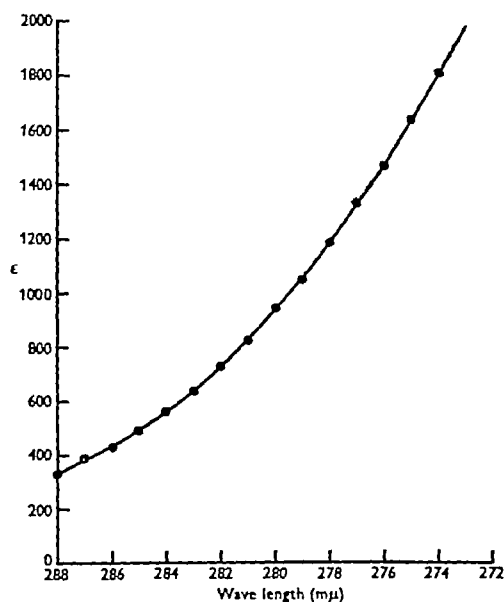


Fig 3 Absorption spectrum of haematinic acid anhydride in water

imide behaves as a monobasic acid with an apparent pK of 4.53. No correction for ionic strength has been made and the value is of the expected order of magnitude. Haematinic acid anhydride behaves as a tribasic acid, but even an approximate calculation of the first and the second pK is not easy. Haematinic acid anhydride may exist in water wholly or partly as acid anhydride which opens instantaneously on addition of alkali. The equilibrium constant for this reaction cannot be calculated from our data. It may suffice therefore to give the mid-points of three steps in the titration, these are as follows 3.75, 5.08, 6.46. It is clear that haematinic acid anhydride is a much stronger acid than the imide and it appeared possible to base a method of separation of the two compounds on this difference.

phase, before and after equilibration with the organic solvent. Methyl ethyl maleic anhydride is insoluble in water and easily soluble in all organic solvents tried. Its removal from an oxidation mixture thus presents no difficulty. The solubility of the two haematinic acids in ether is about the same as in water, and this solvent, which was used by Fischer & Wenderoth (1939), is therefore not convenient for the complete extraction of the imides. This can be done more easily with ethyl acetate, for which distribution coefficients of 15–20 were observed. Haematinic acid anhydride and methyl ethyl maleic acid imide are much more soluble in chloroform than in water, whilst the reverse was found for haematinic acid imide, the distribution coefficient for the latter being about 0.033.

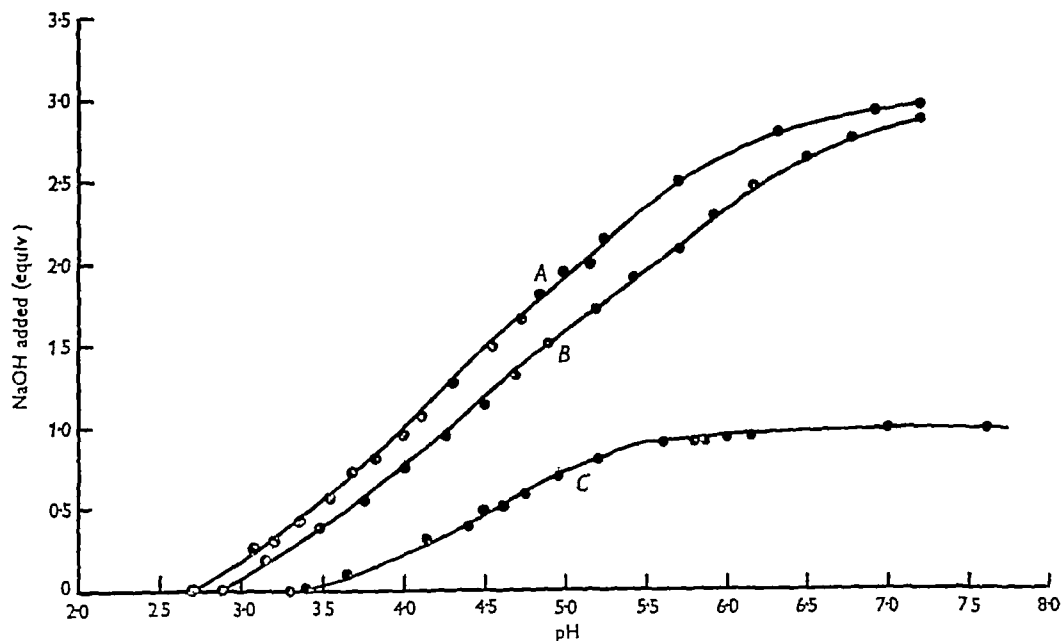


Fig. 4. Electrometric titration of haematinic acid anhydride with (A) and without (B) calcium chloride, and of haematinic acid imide (C).

Fig. 4 also shows that addition of calcium chloride causes an apparent increase in the pK values of haematinic acid anhydride. This effect, due to complex formation, is observed with some other di- and tri-carboxylic acids and can be utilized for the separation of the anhydride from the imide.

Solubilities. Approximate distribution coefficients were determined by shaking aqueous 0.001–0.002M solutions of the two haematinic acids and of methyl ethyl maleic acid imide with an equal volume of organic solvent previously saturated with water. Distribution coefficients were obtained from the difference of ultraviolet absorption of the aqueous

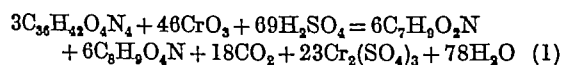
Separation of haematinic acid imide and anhydride

At pH 5.45 about 90% of the imide (pK 4.5) should exist as salt, and 10% as the free acid. However, extraction with ethyl acetate, in which the imide is about 15 times more soluble than in water, should remove more than half of the imide, if the pH is kept constant with a buffer which is not extracted by the organic solvent. The anhydride, on the other hand, should exist at that pH as a mixture of dibasic and monobasic salts, and thus not be extracted to any extent by ethyl acetate. This prediction was put to the test. 9.2 mg of each acid were dissolved in 50 ml 0.5M citrate, pH 5.44, and extracted with ethyl acetate. Spectrophotometric examination of the two aqueous phases showed that about

44% of the imide and almost none of the anhydride was extracted under these conditions. That a smaller amount of imide was extracted than was anticipated is probably due to a decrease in apparent pK of haematinic acid imide in the highly concentrated citrate buffer used. This method was also found to separate oxalic acid and succinic acid, which have been identified as by products of porphyrin oxidation, from haematinic acid imide.

Oxidation of mesoporphyrin methyl ester by chromic anhydride

Under the usual conditions (Fischer & Wenderoth, 1939) the ester is first hydrolysed by 50% (v/v) sulphuric acid and then oxidized by chromic anhydride, after addition of an equal volume of water. The formation of methylethylmaleic acid imide and haematinic acid imide is expressed by the following equation



According to this equation twenty-three O atoms are consumed, six for the oxidation of the methanol and seventeen for that of the porphyrin. Of the six molecules of carbon dioxide produced, two are derived from methanol and four from the methyne groups. In view of the importance of this oxidation for tracer studies generally, it was desirable to establish whether this equation represents the main or only pathway of oxidation.

Stability of maleic acid imides to chromic anhydride and sulphuric acid

Samples (56 mg) of methylethylmaleic acid imide were dissolved in 50 ml of water containing varying concentrations of H_2SO_4 (12.5, 25, 50, % all v/v), and CrO_3 (160 mg). The solutions were kept at 0, 20 and 37° respectively, and 5 ml. samples were withdrawn and CrO_3 was titrated iodometrically. There was no change in the iodine titration, apart from one sample which had been left for 24 hr at 37°. In the latter, the figures indicated that about 4% of the CrO_3 had been consumed. Similar results were obtained with haematinic acid imide. The possibility that the acid might hydrolyse the imides to anhydrides was also investigated. Haematinic acid imide (10.2 mg) was dissolved in 50% (v/v) H_2SO_4 (10 ml), 1 ml. was diluted at once with water to 10 ml. and the ultraviolet absorption measured from 285 to 310 $m\mu$. After standing for 3.5 hr at 23°, 1 ml. was again withdrawn, diluted to 10 ml. and its extinction curve measured. There was no change and the values agreed closely with those expected for the imide. Another series of experiments was carried out as follows: haematinic acid imide (150 mg) was dissolved in water (35 ml), to 5 ml. portions were added, 1, 2, 3, 4 and 5 ml. respectively of a 50% (v/v) H_2SO_4 , and the volume was made up with water to 10 ml. These solutions were allowed to stand either at 0° or at 23° for 4 hr., and were then extracted 3 times with ethyl acetate (20 ml. each), which removed the unchanged imide. Estimation of NH_3 in the aqueous fractions in a Kjeldahl apparatus showed that no NH_3 had been formed. It is concluded, therefore, that the two imides are

stable, both to the oxidative action of CrO_3 , and to the hydrolytic effect of H_2SO_4 under the conditions used for the oxidation of porphyrins.

Oxidation of mesoporphyrin This was investigated by following the consumption of chromic anhydride, the production of carbon dioxide, and the formation of the two imides and other possible compounds.

In a typical experiment, mesoporphyrin methyl ester (250 mg) was dissolved in 50% (v/v) H_2SO_4 (15 ml) and the solution shaken for 2 hr at 18–20°. The solution, now containing free mesoporphyrin, was cooled to 0°, and CrO_3 (725 mg, 10% excess) dissolved in 15 ml water previously cooled to 0°, was added. The mixture was then shaken at about 18°, whilst N_2 , freed of CO_2 , was passed through. CO_2 evolved was collected in three flasks containing measured amounts of 0.1N $Ba(OH)_2$. After suitable intervals the $Ba(OH)_2$ solutions were removed, acetone (0.8 vol.) added and the $Ba(OH)_2$ titrated with 0.1N HCl , using thymol blue as internal indicator. After 5 hr the mixture was extracted three times with 30 ml portions of ethyl acetate, the combined extracts were washed with saturated $NaCl$ (5 ml) and the salt solution again extracted with ethyl acetate (10 ml). The combined ethyl acetate solutions were dried and the solvent removed *in vacuo*. The weight of the residue was 201.4 mg. The yield at this stage would be 75%, if the residue consisted entirely of the two imides. The aqueous fractions were used for estimation of total N and NH_3 . The residue from the ethyl acetate extractions was dissolved in boiling water (10 ml.) and filtered. After cooling to 0°, the pH was brought to 7.0 by addition of $NaHCO_3$ (100 mg). The solution was now extracted with $CHCl_3$ (3×30 ml) and the combined $CHCl_3$ solutions were washed with saturated $NaCl$ and dried. On removing the solvent, 71.6 mg of almost pure methylethylmaleic acid imide were obtained. This was dissolved in a little hot water and crystallized by slow evaporation in a partially evacuated desiccator. There were obtained 58.5 mg of the imide of m.p. 68° (Found N (Kjeldahl) 10.2, calc. for $C_7H_5O_2N$ N, 10.1%). Yield of crude imide was 63% of the theoretical. The neutral aqueous solution was acidified with N HCl (0.5 ml.) to pH 5 and 20 ml. of 0.5M citrate buffer, pH 5.44, were added. The solution was extracted with ethyl acetate (4×65 ml). The combined ethyl acetate solutions were extracted twice with 10 ml. portions of N HCl containing 10% (w/v) $NaCl$ and once with saturated $NaCl$ (5 ml.). The combined aqueous solutions were again extracted with ethyl acetate (10 ml). From the combined ethyl acetate solutions there were obtained 116.7 mg of a partly crystalline solid. Ultraviolet absorption of an aqueous solution of this material revealed the presence of some material absorbing at about 300 $m\mu$, other than haematinic acid imide or anhydride. The solid was therefore dissolved in water (10 ml.), extracted twice with $CHCl_3$ (1 ml.) to remove impurities and then with ethyl acetate (3×20 ml). On concentrating the dried ethyl acetate solutions, crystals were obtained which were recrystallized from a very small amount of water, giving 80.5 mg of imide of correct melting point analysis and ultraviolet absorption spectrum. In a few experiments the material was still not quite pure and it was then sublimed at 0.001 mm pressure at a bath temperature of 150–155°. The oxidation and separation were carried out with 50 mg of mesoporphyrin methyl ester and the yields of the two imides were only slightly lower. In several experi-

ments it was desired to measure the CrO_3 consumption, this was done by removing samples from the oxidation mixture, at intervals, and titrating the CrO_3 iodometrically

The results of the various estimations may be described as follows at 0° oxidation was very slow, partly at least owing to the insolubility of the porphyrin. The oxidation appeared to be complete at $18-20^\circ$ within 5 hr, after that time there was no further evolution of carbon dioxide or uptake of oxygen. The mesoporphyrin disappeared during the first 2 hr, as shown by spectroscopic examination. However, figures for carbon dioxide evolution or chromic anhydride consumption never reached the values required by equation (1). In several experiments it was found that between 68 and 75% of the theoretical amount of carbon dioxide was produced in the first 1.5 hr, and after 5 hr the values were from 82 to 86%. The oxygen uptake showed a similar course, about 85% being observed after 5 hr. The oxidation of methanol was examined by following carbon dioxide evolution and chromic anhydride consumption, and was found to be similar to that of mesoporphyrin ester. The 5 hr values were slightly higher and varied between 85 and 94% of the theoretical. The two imides could never be obtained in a yield higher than 66%, and the mechanical losses as judged from recovery experiments are not likely to exceed 5%. Moreover, it was found that 12-16% of the total nitrogen was present in the first aqueous fraction, not extractable by ethyl acetate. This nitrogen appeared as ammonia on mild treatment with alkali and must therefore be present either as ammonia or as a very labile amide. This observation, and the reported presence of volatile acids, maleic acid anhydrides and of oxalic and succinic acids in the oxidation products of porphyrins (Küster, 1922; Fischer & Wenderoth, 1939), leads to the conclusion that either the intermediate in the oxidation is more sensitive to oxidative or hydrolytic attack than the imides, or that there is a side reaction not leading through the maleic acid imides. This oxidative reaction cannot account for more than 15% of the mesoporphyrins, as judged from the amount of ammonia produced.

However, if this more complete oxidation were the only side reaction, it would be expected that the values for oxygen consumption and carbon dioxide evolution should be higher than those predicted by equation (1). In fact, the figures were lower by 14-18% than those calculated. Since the mesoporphyrin has disappeared at the end of the oxidation it would follow that another reaction must occur, giving products, possibly dipyrrolyl derivatives, which are relatively resistant to further oxidation by chromic anhydride. This would also explain the presence of material giving an intense absorption at $300 \text{ m}\mu$ in the haematinic acid fraction. It was also observed that the nitrogen content of this fraction

was much higher than could be accounted for by the haematinic acid imide. It can thus be concluded that 63-70% of the oxidation products of mesoporphyrin consist of methylethylmaleic acid imide and haematinic acid imide, the rest being accounted for by more complex substances of unknown structure, and secondly, by more completely oxidized substances arising from a side reaction.

These results have an important bearing on degradation experiments in tracer studies of porphyrins, both in this investigation and in other studies under way. The porphyrin structure contains two pyrrolenine, one pyrrol and one maleic acid imide nucleus. The three structures may be expected to give rise on oxidation with chromic anhydride to varying amounts of maleic acid imide derivatives, and if bond fixation were to occur (cf. Fischer & Orth, 1937) it would not be permissible to assume that methylethylmaleic acid imide was derived to an equal extent from rings I and II, and haematinic acid imide from rings III and IV. In neutral solution tautomerism is possible since only two of the four nitrogen atoms carry hydrogens. However, oxidation takes place in strongly acid solution, in which the mesoporphyrin is presumably present as bivalent cation. In such a structure tautomerism is impossible (see Corwin, 1943) and we may suppose that complete resonance occurs. We assume that all three structures make similar contributions to the four nuclei, and that the two imides are derived to an equal extent from rings I and II or III or IV respectively. In any case, the fact that the two imides could be isolated in yields well above 50% indicates clearly that each of the two compounds cannot be derived from one nucleus only.

Another, less theoretical, point concerns the source of the carbon dioxide evolved during the oxidation. The major part must arise, as required by equation (1), from the four methyne and the two methanol carbon atoms. However, the side reaction which produces ammonia and volatile acids will presumably yield some carbon dioxide from the pyrrolic nuclei in addition to that derived from methyne carbon atoms. From the amount of ammonia present in the aqueous phase it can be estimated that this reaction cannot account for more than 15% of the mesoporphyrin oxidized. From this calculation it follows that about 10% of the carbon dioxide evolved may arise from carbon atoms other than the methyne and methanol carbon atoms. On this basis approximately 60% of the carbon dioxide evolved may be ascribed to the methyne carbon atoms, 30% to the methanol, and 10% to unknown sources.

Biological experiments

Preparation of isotopic compounds Glycine containing 31.69 atom % excess ^{15}N was prepared from phthalimide and ethyl chloroacetate (Schoenheimer & Ratner, 1939), and

ethanolamine hydrochloride with 31.65 atom % excess ^{15}N , as described by Bloch & Schoenheimer (1941)

Administration of compounds and care of animals Male and female Hollingsworth half lopped rabbits (1.5–1.8 kg body weight), and rats of the black and white Institute strains, (280–320 g body weight) were used. The rabbits were fed the ordinary stock diet, whilst the rats, from 5 days before the administration of the isotopic compounds, were given a semi synthetic diet with a protein content of 5%. Two days after the feeding of the labelled substances had finished, the rats were put back on the stock diet containing 18% protein. The animals were kept in metabolism cages of the usual design. The isotopic compounds were fed on 5 successive days in equal doses to animals which had been fasting for about 18 hr. The glycine and ethanolamine hydrochloride were dissolved in water to give a 5% solution which was used to moisten a weighed quantity of the dry, pelleted food, representing about 40% of the normal daily ration. The animals usually consumed the food within 2–3 hr and any scattered pellets were collected and weighed. The correction for this loss was negligible in almost all experiments. Each of 5 rabbits consumed altogether 0.6 mmol glycine/100 g body weight. Four rats were each given 0.8 mmol ethanolamine/rat, whilst three rats received glycine, the dose amounting to 0.6 mmol/rat. The animals were killed on the fifteenth or seventeenth day after the last administration of the isotopic compound.

Isolation of mesoporphyrin The rabbits were anaesthetized with ether, and blood collected from the aorta. In several experiments the animals were perfused through the vena cava with heparin containing saline. The heparinized blood was centrifuged, the red cells were twice washed with saline, and then lysed by addition of an equal volume of water containing saponin. In the earlier experiments haemin was isolated by adding the haemoglobin solution slowly, at 95°, to 5 vol glacial acetic acid containing 1% (v/v) saturated NaCl solution. The solution was allowed to cool very slowly to room temperature and was then left at 0° for 18 hr. The haemin crystals were separated and washed with acetic acid, water, ethanol and ether. Haemin was then dissolved in methanol containing 10% (w/v) anhydrous oxalic acid, about 10 ml being used for 50 mg haemin. The further procedure followed that of Grinstein (1947). The protoporphyrin methyl ester was recrystallized, if the melting point was lower than 220°. In later experiments the method of Grinstein was used from the beginning.

The reduction of protoporphyrin to mesoporphyrin ester was done either by the HI method of Grinstein & Watson (1943), or by a catalytic method similar to that of Granick (1948), who used colloidal Pd. In agreement with other workers, it was observed that, with the usual preparations of Pd or Pt catalysts, no reduction to mesoporphyrin took place. However, a catalyst of the polyvinyl type described by Rampino & Nord (1941) was found to be effective. Reduction by the latter method, which gave slightly higher yields than the HI method, was done as follows. 0.276 g of polymerized methyl methacrylate (average mol. wt 1×10^5) was dissolved in anhydrous formic acid. The latter had been dried over CuSO_4 distilled *in vacuo*, and crystallized at 0°. PdCl_2 solution (0.25 ml), containing 15 mg Pd, was then added. H_2 was passed in for 3–4 min and NaHCO_3 (0.045 g) in water (0.25 ml) added. As soon as all the Pd had been reduced (about 1 min) protoporphyrin methyl ester (0.15–0.2 g) was added. The solution was kept at 52–55°, while a slow stream

of H_2 was passed through. When reduction was complete, as judged spectroscopically, the solution was poured into ether. The methyl methacrylate, which precipitated, was separated by decantation and repeatedly extracted with ether. The combined ether solutions were extracted with N HCl and the latter with CHCl_3 . The CHCl_3 solution was washed with water, dried, and concentrated *in vacuo*. The mesoporphyrin ester, which was crystallized from CHCl_3 , methanol, was usually obtained in a yield of 70%, and had m.p. 212–214°. The oxidation to the maleic acid derivatives was carried out as described above.

In the experiments with rats no degradation was carried out, haemin was isolated as described above, and recrystallized by dissolving in pyridine CHCl_3 and precipitating with hot glacial acetic acid containing NaCl. The isotope ratio was estimated with the mass spectrometer.

RESULTS AND DISCUSSION

Table I shows that the ^{15}N contents of the methyl-ethylmaleic acid imide and of the haematonic acid imide fractions are almost identical in all six experiments. With only one rabbit (no. 1) is the difference

Table I. ^{15}N contents of methyl-ethylmaleic acid imide and of haematonic acid imide obtained from the haemoglobin of rabbits fed isotopic glycine

(Glycine contained 31.69 atom % excess ^{15}N)

Rabbit no	Sex	Atom % excess ^{15}N		
		In acidic fraction	In non acidic fraction	Difference
1	M	0.108	0.120	0.012
2	F	0.186	0.190	0.004
3	M	0.086	0.092	0.006
4	M	0.122	0.117	0.006
5	F	0.156	0.150	0.006
6	M	0.142	0.139	0.003

outside the limits of experimental error, but it is doubtful whether this isolated observation is significant. We may, therefore, conclude that the labelled nitrogen enters at a similar rate into the acidic and non acidic nuclei of the haemin group. Haemoglobin contains four haemin groups and each haemin has four pyrrolic nuclei. However, the experiments reported here do not conclusively prove that all sixteen pyrrolic nuclei in a haemoglobin molecule contain similar amounts of ^{15}N . In the Experimental section of this paper arguments were adduced to show that the two imides are derived to an equal extent from rings I and II, and III and IV, respectively. Even if this is accepted, however, it is still possible that, say, rings I and III contain all the excess ^{15}N , whilst rings II and IV contain none. This possibility, although it may appear remote, cannot be excluded on the basis of the data reported here. However, unpublished results show that, taking into account the rate of formation of haemoglobin and the ^{15}N content of the glycine pool, the isotope

content in haemin is the highest theoretically possible. We may, therefore, accept the conclusion that all four pyrrolic nuclei of any one haemin group and all four haemin groups of the haemoglobin molecule have identical or closely similar isotope ratios. From this it follows that the four rings either arise from a common pyrrolic precursor, which is modified afterwards, or that the four rings are synthesized independently at similar or identical rates from glycine or a substance derived exclusively from glycine.

The experiments with rats are shown in Table 2. The ^{15}N contents of the haemin after feeding a rather small amount of isotopic glycine are of the expected

Table 2 ^{15}N contents of haemin of rats which had received isotopic glycine or ethanolamine

(Glycine and ethanolamine each had 31.69 atom % excess ^{15}N)

Rat no	Sex	Substance fed	Atom % excess ^{15}N of haemin
1	M	Ethanolamine	0.006
2	F	Ethanolamine	0.022
3	M	Ethanolamine	0.016
4	F	Ethanolamine	0.018
5	M	Glycine	0.102
6	F	Glycine	0.105

order of magnitude. With ethanolamine, which was fed at a slightly higher molar concentration, only a small amount of incorporation of ^{15}N is observed. It is clear that ethanolamine cannot be an intermediate between glycine and protoporphyrin. The small

amount of ^{15}N present in these experiments may be due to the fact that ethanolamine can be converted, probably largely through choline and betaine, to glycine (Stetten, 1941).

SUMMARY

1. A synthesis of methylethylmaleic acid imide and of haematinic acid imide, two oxidation products of mesoporphyrin, is described. The ultraviolet absorption spectra in water and the dissociation constants of these and related compounds have been determined.

2. The oxidation of mesoporphyrin by chromic anhydride was investigated, and conditions were established which permit the isolation of the two maleic acid imides on a micro scale. This method was applied to the degradation of mesoporphyrin obtained from rabbits fed glycine containing an excess of ^{15}N . The results show that the acidic and non acidic pyrrole nuclei in haemin have the same or closely similar isotope ratios.

3. Ethanolamine containing an excess of ^{15}N was fed to rats. The haemin obtained had a much lower ^{15}N content than that of control animals which had received a slightly smaller amount of labelled glycine. It is concluded that the nitrogen of ethanolamine is not used to any marked extent in the synthesis of protoporphyrin.

The authors wish to thank Dr R. Bentley, Miss M. G. French and Mr G. Dickinson for mass spectrometric analyses and Dr W. J. Elford and Dr G. Popják for help in some experiments.

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Factors Affecting Synthesis of Ascorbic Acid in Cress Seedlings

2 ASCORBIC ACID SYNTHESIS IN RELATION TO SUGAR FORMATION

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(Received 21 February 1949)

In a previous paper (Mapson & Cruickshank, 1947) it was shown that certain salts stimulated the synthesis of ascorbic acid in cress seedlings and increased the pH of the cell sap, whereas other salts had the reverse action. It was concluded from this that the deciding factor was the amount of base made available to the plant after metabolism of the salt.

Ray (1934) found that certain sugars increased the production of ascorbic acid when supplied to the excised pea embryo. He calculated his results on the basis of concentration, but, as growth was affected by the various treatments, we have recalculated them as the amount produced by a given number of seedlings. Of all the substances tested by him, hexose sugars alone produced a significant increase in ascorbic acid. Of these fructose was the most effective, followed in descending order by mannose, glucose and galactose. He suggested, therefore, that the hexose sugars served as precursors of ascorbic acid.

In view of Ray's (1934) results it seemed desirable to ascertain whether the treatments described in our earlier paper (Mapson & Cruickshank, 1947) had affected the synthesis of ascorbic acid by reason of their effect on the formation of hexose sugars. We therefore followed the formation of hexoses and ascorbic acid in seedlings germinated in the presence of various salts. The salts chosen fell into three groups, viz those which had been shown (a) to decrease the synthesis, (b) to increase the synthesis, and (c) to have little effect.

METHODS

Conditions of germination

The seeds were germinated under the conditions described in the earlier paper (Mapson & Cruickshank, 1947). In the present experiments, when sugar and a nitrogenous salt were added, it was found essential to add HgCl_2 (1/20,000) to the medium to prevent the growth of moulds. The Hg salt was also added to the controls. The seedlings were grown in the dark, to eliminate changes due to light or photosynthetic activity.

As in our previous communication all results are expressed in terms of the amounts of the individual constituents

produced by 100 seedlings, this having been found to be a more satisfactory basis for comparison than concentration.

Estimation of sugars

When sugars were added to the medium in which the seeds were grown, all samples of seedlings taken for sugar analysis were washed in several changes of distilled water to remove any adhering sugar. Sugars were extracted from the seedling with boiling 80% ethanol, the ethanol removed under reduced pressure and the residual liquid cleared by treatment with neutral Pb acetate, excess of Pb was removed by oxalate.

Total reducing sugars. The total reducing sugars were estimated in accordance with the method of Somogyi (1937) as modified by Hanes and described by Hockenhull & Herbert (1945). The reducing power has been expressed in equivalents of glucose.

Non-fermentable sugars. The non fermentable reducing sugars and other non fermentable reducing substances were estimated after removal of the fermentable sugars by yeast, as follows: 10 g of baker's yeast ground with 100 ml of water were allowed to stand 1 hr at room temperature. The solution was centrifuged and the residue washed with a further 100 ml of water and recentrifuged. The washed yeast was taken up in 100 ml of phosphate buffer (0.05M, pH 6.0). To 10 ml of the sugar solution, 5 ml of the yeast suspension were added. The combined solutions were incubated for 3 hr at 37°, cooled and filtered through a fine paper and a measured volume taken for estimation of reducing titre.

Hexose sugars. Partition chromatography showed that the only hexose sugars present were glucose and fructose (Jermyn & Isherwood, 1949). These were calculated as being equivalent to the total reducing sugars minus the non fermentable fraction ($\text{T.R.S.} - \text{N.F.}$).

Sucrose. Sucrose was estimated by determining the increase in reducing titre after hydrolysis by invertase. In the following Figures and Tables the value for sucrose is expressed in terms of its glucose equivalent.

Fructose. Fructose was estimated directly by the resorcinol method proposed by Roe (1934) and modified by Cole (1942, unpublished).

'Glucose' The glucose content has been calculated as being equivalent to the hexose sugars minus fructose.

Ascorbic acid

Ascorbic acid was estimated by titration against 2,6-di-chlorophenolindophenol, as in our previous investigations (Mapson & Cruickshank, 1947).

Fat (ether extract)

The seedlings were thoroughly ground up with quartz, dried with Na_2SO_4 and extracted with ethyl ether for 6 hr. The ether was evaporated and the residue taken up in light petroleum. Na_2SO_4 was again added to eliminate traces of water, the solution was filtered, and the light petroleum removed under reduced pressure. Acid values and iodine values were determined on the ether extract, the latter by Wijs's method.

pH of cell sap

The same technique was used as described by Mapson & Cruickshank (1947)

RESULTS

The food reserves of the cress seed

The cress (*Lepidium sativum*) belongs to the Cruciferae. Its seeds contain about 26% of fat as their main food reserve. A small amount of sucrose is also present, but there is no starch or hexose sugar. Expressed on the basis of 100 seeds, the fat and carbohydrate reserves were as follows:

	(mg)
Fat	55-60
Sucrose	10
Non fermentable sugars, etc	6.5

Changes in seeds germinated in water

When cress seeds were germinated in the dark in distilled water, the following changes were observed (Fig 1). There was a rapid fall in sucrose, which

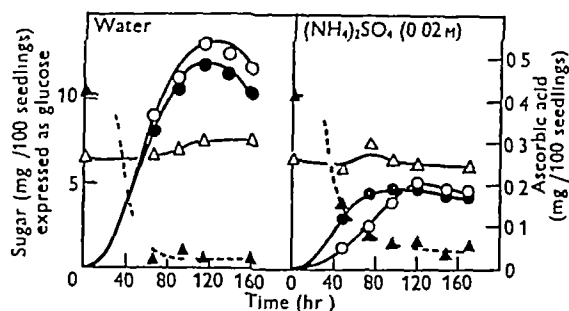


Fig 1 Ascorbic acid and sugar content of seedlings germinated in water and $(\text{NH}_4)_2\text{SO}_4$, ▲—▲, sucrose, ○—○, hexose (T R S - N F), △—△, non fermentable sugars, ●—●, ascorbic acid

reached a constant value at approximately 48 hr. During this period an appreciable amount of ascorbic acid was synthesized, but little or no fat had been mobilized (see Fig 9). The non-fermentable sugars

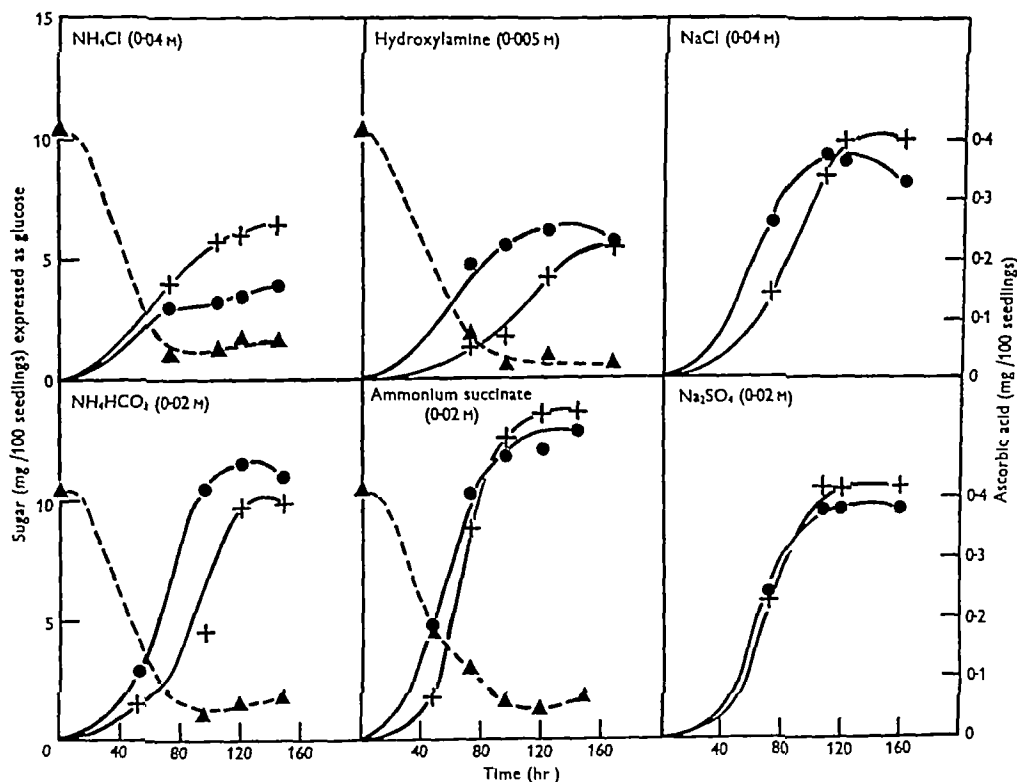


Fig 2 Ascorbic acid and sugar content of cress seedlings germinated in ammonium and other salt solutions, ●—●, ascorbic acid, +—+, hexose (T R S - N F), ▲—▲, sucrose

remained at 6.5–7 mg/100 seedlings throughout the experimental period. The greatest change was in the hexoses, which reached a maximum at approximately 120 hr of germination. The most significant observation was the close correspondence between the rise in hexose sugars and the rise in ascorbic acid.

maximum amount of hexose sugar and of ascorbic acid formed was, respectively, only 36 and 39 % of that formed in seedlings grown in water.

Similar experiments were carried out with NH_4Cl and $\text{NH}_2\text{OH HCl}$ (Fig 2), both of which decrease the formation of ascorbic acid and hexose sugars.

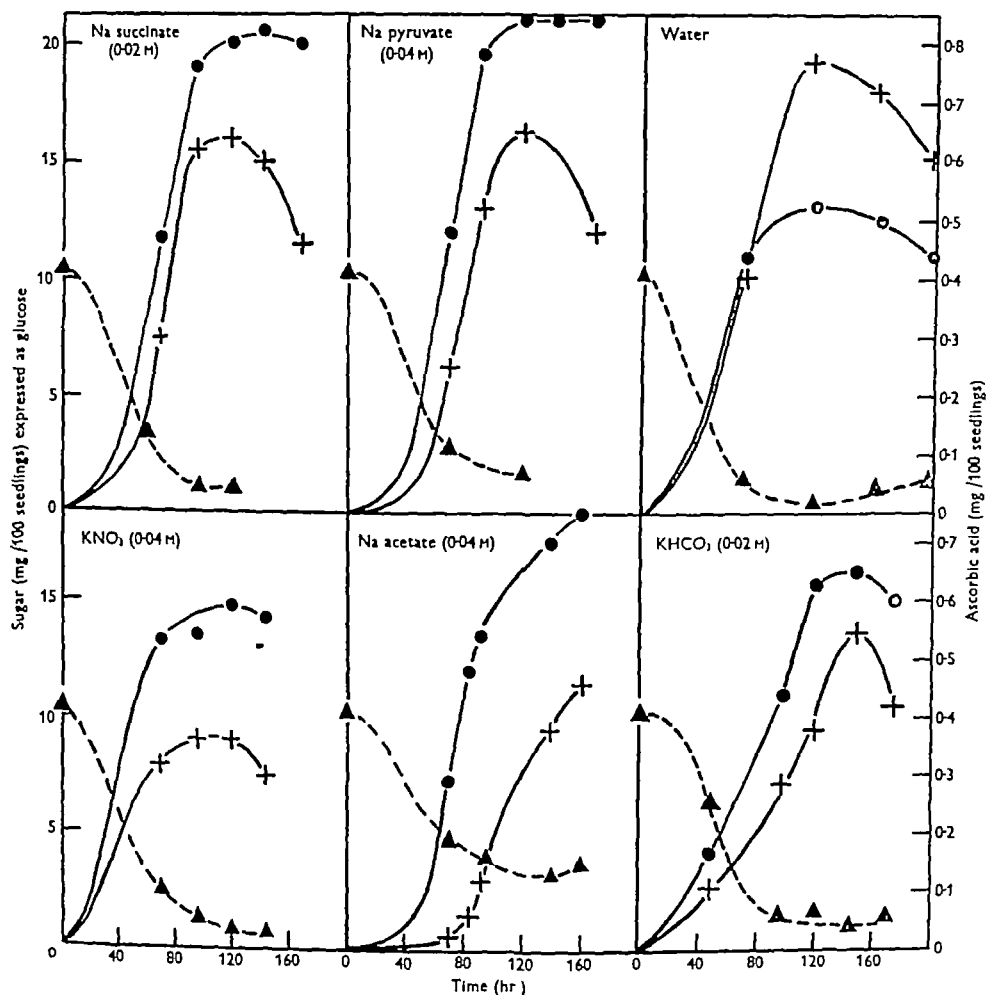


Fig 3 Ascorbic acid and sugar content of cress germinated in sodium and potassium salts, ●—●, ascorbic acid, +—+, hexose (T R S — N F), ▲—▲, sucrose

Changes in seeds germinated in different ammonium salts

A similar experiment was carried out with seeds germinated in $(\text{NH}_4)_2\text{SO}_4$ (Fig 1). Here, also, sucrose rapidly disappeared. The fall was slightly less rapid than in the seedlings grown in water, but this difference has not invariably been observed. The non-fermentable sugars again showed no change. The chief difference was the much smaller amount of hexoses formed, and thus was associated with a correspondingly reduced synthesis of ascorbic acid. The

The changes found with NH_4Cl were essentially similar to those observed with $(\text{NH}_4)_2\text{SO}_4$, but with $\text{NH}_2\text{OH HCl}$ the correspondence between the rates of production of these constituents was less close.

Our original experiments had shown that with ammonium salts such as the bicarbonate and succinate where both ions may be utilized by the plant, the synthesis of ascorbic acid is similar to that in seedlings grown in distilled water, but greater than in seedlings grown in $(\text{NH}_4)_2\text{SO}_4$ or NH_4Cl . This increased synthesis of ascorbic acid is associated with an increased formation of hexoses (Fig 2).

Changes in seeds germinated in different sodium and potassium salts

When combined with a 'utilizable' anion, Na and K salts stimulate the synthesis of ascorbic acid, when the anion is non-utilizable, ascorbic acid is decreased (Mapson & Cruickshank, 1947). Reference to Figs 2 and 3 again shows that with various Na and K salts, the formation of hexoses is closely related to that of ascorbic acid. Non utilizable ions such as Cl^- and SO_4^{--} , when combined with ammonium, had a more depressing effect on both constituents than when combined with Na or K (cf Figs 1 and 3).

Changes induced by supplying glucose

Thus parallelism between hexose sugars and ascorbic acid supports Ray's (1934) hypothesis that ascorbic acid may be derived from such sugars. It

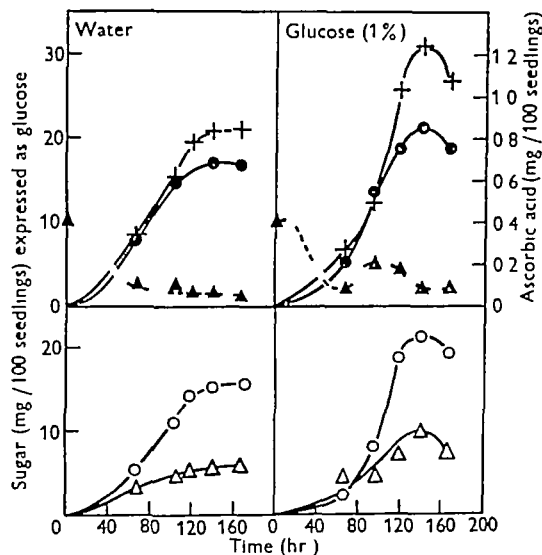


Fig 4 Ascorbic acid and sugar contents of seeds germinated in water and glucose (1%) solution, ▲—▲, sucrose, +—+, hexose (T R S - N F), ●—●, ascorbic acid, ⊙—⊙, glucose, △—△, fructose

was therefore to be expected that increasing the latter in the seedling would increase the synthesis of ascorbic acid. To test this view cress seedlings were germinated in water and in a solution of 1% (w/v) of glucose (Fig 4). During the first 90 hr the amount of hexose sugars was similar in both cases, which indicated that in the early stages of germination little or no sugar had been absorbed. After this period the seedlings supplied with glucose contained considerably more hexose sugar, and also more ascorbic acid. Fig 4 also shows that part of the glucose supplied to the seedlings was converted into fructose, since the latter was proportionately increased when glucose was supplied. It is possible

that one or other, or both of these sugars may act as precursors of ascorbic acid, but the present data do not elucidate this point.

The results of a similar experiment in which glucose was supplied to seedlings growing in $(\text{NH}_4)_2\text{SO}_4$ are illustrated in Fig 5. The addition of glucose slightly delayed the hydrolysis of sucrose and increased considerably the amount of hexose sugar, but the rise in ascorbic acid was relatively slight. The conversion to fructose of the absorbed glucose was not impaired under these conditions. A similar result was observed when NH_4Cl was substituted for $(\text{NH}_4)_2\text{SO}_4$. The fact that it was possible under these conditions to increase the hexose content of the seedlings to a value similar to that in seedlings grown in water, without at the same time appreciably increasing the ascorbic acid, suggested that the depressing effect of $(\text{NH}_4)_2\text{SO}_4$ or NH_4Cl cannot be due simply to a reduction in the amount of hexose sugars, or to a failure to convert glucose into fructose.

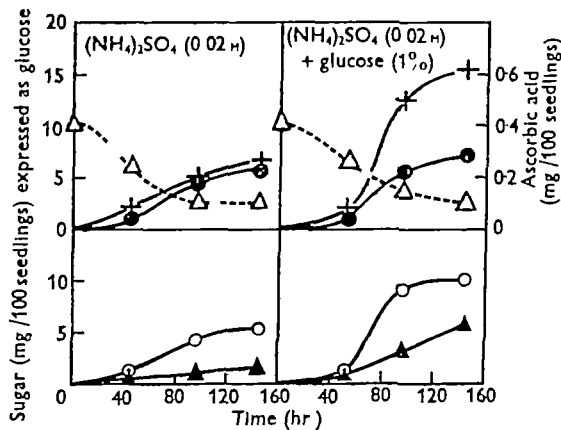


Fig 5 The ascorbic acid and sugar content of cress seedlings germinated in presence of $(\text{NH}_4)_2\text{SO}_4$ and glucose, △—△, sucrose, +—+, hexose (T R S - N F), ●—●, ascorbic acid, ▲—▲, fructose, ⊙—⊙, glucose

When glucose was supplied to seedlings growing in solutions of Na salts which favour the synthesis of ascorbic acid, e.g. succinate or pyruvate, the increase in the hexose sugar was associated with a greater increase in ascorbic acid. The relationship between ascorbic acid and hexose formation in these and the foregoing experiments is shown more clearly in Fig 6A, B. Here the ascorbic acid is plotted against the hexose, the curves all pass through the origin, but have gradients which appear to be determined by the nature of the salt used. The salts fall into three distinct groups. Na or K salts containing a utilizable anion give curves with the steepest gradients (group 1), water alone or ammonium salts containing a utilizable anion give curves with an intermediate gradient (group 2), whilst ammonium salts con-

taining a non utilizable anion give curves in which the gradient is still further reduced (group 3)

Furthermore, when seedlings growing in these various salt solutions are supplied with glucose, the formation of ascorbic acid is increased by an amount

with high pH and the less steep with low pH of the cell sap. In all experiments there was a drift in the pH of the cell sap to more alkaline values as growth of the seedling increased, but this did not appear to alter the quantitative relation between ascorbic acid and hexose

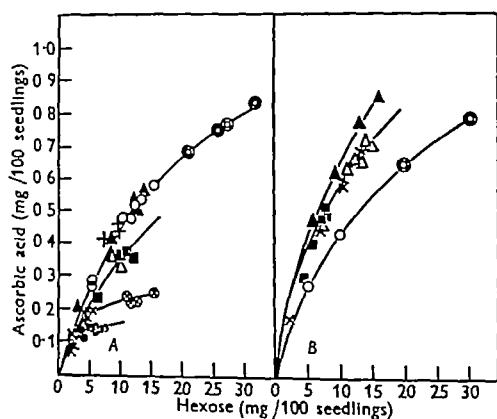


Fig 6 The relation between ascorbic acid and hexose for maton as affected by different salts A, \circ , water, \bullet , NH_4Cl (0.04M), \times , $(\text{NH}_4)_2\text{SO}_4$ (0.02M), \blacksquare , Na_2SO_4 (0.02M), \triangle , NaCl (0.04M), $+$, NH_4HCO_3 (0.02M), \blacktriangle , ammonium succinate B, \circ , water (curve for comparison), \triangle , sodium succinate (0.02M), \times , KHCO_3 (0.02M), \blacksquare , KNO_3 (0.04M), \blacktriangle , sodium pyruvate (0.04M) Ringed symbols represent values obtained when D glucose was supplied in addition to salt

which is determined by the gradient of the curve, the points corresponding falling on the extrapolated curve. Thus, with salts in group (1) the increased synthesis of ascorbic acid induced by increasing the hexose sugar of the seedling is relatively greater than with salts of group (2) and very much greater than with salts of group (3)

The simplest interpretation of such data is that ascorbic acid is formed directly from hexose, and that, as compared with seedlings grown in water, this conversion is stimulated by salts of the first group, is unaffected by salts of the second group and impaired by salts of the third

As was pointed out in an earlier paper (Mapson & Cruickshank, 1947) these three classes of salts differentially affect the pH of the cell sap. Thus, compared with seedlings grown in water, salts in group (1) increase the pH of the cell sap, those in group (2) do not appreciably alter it, whilst those in group (3) reduce it. These changes in pH have been studied in more detail in this work. The data (Fig 7) show the changes in pH during germination of seedlings grown in water, and in solutions of salts representative of the three groups mentioned above. The gradients of the curves showing the relation between ascorbic acid and hexose sugars form a well defined series in which the steepest are associated

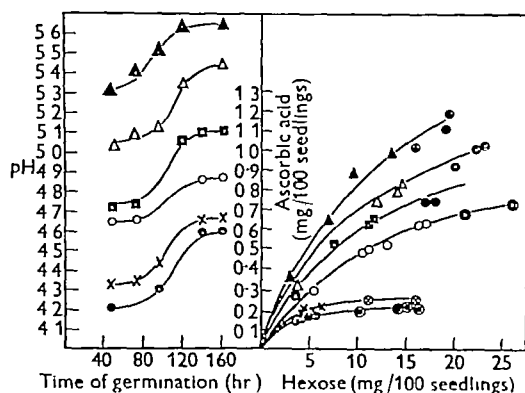


Fig 7 The effect of different salts on the pH of the cell sap and the relation between ascorbic acid and hexose in cress seedlings temp 20° , \bullet , NH_4Cl (0.04M), \times , $(\text{NH}_4)_2\text{SO}_4$ (0.02M), \circ , water, \square , ammonium succinate (0.02M), \triangle , sodium succinate (0.02M), \blacktriangle , sodium pyruvate (0.04M) Ringed symbols represent values obtained when D glucose was supplied in addition to the salt

Sugars other than glucose

Since the foregoing evidence supported the view that ascorbic acid is formed from hexose sugars, it was considered desirable to ascertain whether other sugars could similarly be converted into ascorbic acid. The effect of supplying pentoses and hexoses other than glucose was therefore investigated. L Arabinose, D xylose and D ribose, in concentrations of 0.5 and 1% were without effect on ascorbic acid, although quite considerable amounts were absorbed by the seedling, as shown by the increase in the non fermentable sugar fraction. Sucrose, D-fructose and D galactose each increased the synthesis to about the same extent as glucose. On the other hand, D mannose depressed synthesis and also adversely affected growth, its influence being proportional to the concentration employed.

Mannose supplied in combination with other sugars

The marked inhibition of the synthesis of ascorbic acid induced by supplying D mannose appeared to be of sufficient importance to warrant further study, and the following experiment was carried out. The results (see Fig 8) illustrate the effect on the synthesis of ascorbic acid of supplying different concentrations of glucose in combination with 0.5% of mannose. It will be seen that the production of

ascorbic acid was not affected during the first 50 hr. Subsequently the inhibitory effect of mannose decreased progressively as the concentration of glucose increased. Similar results were obtained when galactose or fructose was substituted for glucose.

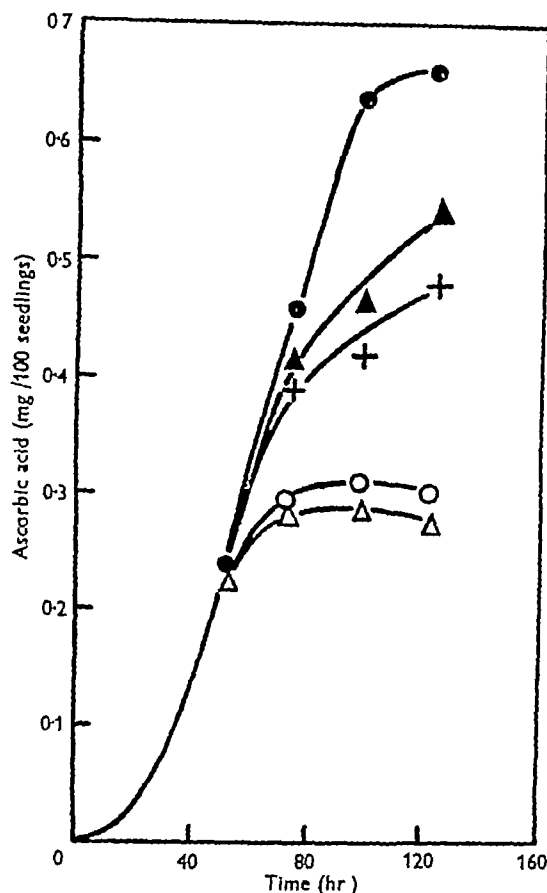


Fig 8 Effect of mannose together with glucose, ●—●, water, ▲—▲, mannose (0.5%) + glucose (1%), +—+, mannose (0.5%) + glucose (0.5%), ○—○, mannose (0.5%) + glucose (0.25%), △—△, mannose (0.5%)

Changes in fat during germination

The poor development of hexose sugars in seedlings germinated in solution of salts like $(\text{NH}_4)_2\text{SO}_4$ prompted us to investigate whether such salts affected the production of these sugars by reason of their influence on the mobilization of the reserves of fat of the seedling. The data obtained, although throwing no further light on the main problem with which this paper is concerned, were nevertheless of sufficient interest to be included here.

Reference to Fig 1 shows that hexose sugars are formed in the seedling during germination in amounts exceeding those which could be produced from the sucrose present in the seed. Since under the conditions of these experiments photosynthesis was

precluded, such sugars must be derived mainly from the reserves of fat which the seed contains. The changes in the amount of fat present in seedlings grown in water and in $(\text{NH}_4)_2\text{SO}_4$ are shown in Fig 9. During the first 48 hr there was little or no reduction in the amount of fat, but, as growth proceeded, it steadily decreased. The rate of mobilization was more rapid in seedlings grown in water than in those grown in $(\text{NH}_4)_2\text{SO}_4$, the fat content at the end of 168 hr falling from 57 mg to 20 and 30 mg respectively. On the assumption that the average

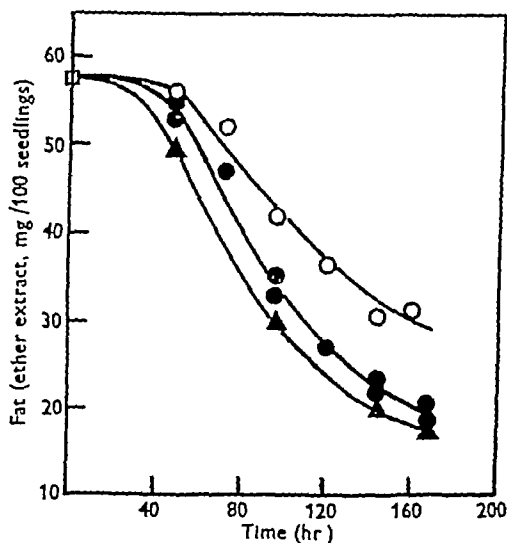


Fig 9 Changes in fat content of seedlings during germination, □, dry seed, ○—○, seeds germinated in $(\text{NH}_4)_2\text{SO}_4$ (0.02M), ●—●, seeds germinated in water, ▲—▲, seeds germinated in KNO_3 (0.02M)

number of carbon atoms in the triglycerides is 20, and that these can be quantitatively converted to hexose, 10 mg of fat (i.e. the maximum observed difference) could lead to the production of approximately 20 mg of hexose sugar, which is much greater than the difference observed between the amount of hexose formed in seedlings grown in water and in $(\text{NH}_4)_2\text{SO}_4$. In those instances in which rather more hexose sugar was formed, e.g. in seedlings grown in KNO_3 or in water, there was some evidence that fat was mobilized rather more rapidly.

These results suggest that the decrease of hexose sugars in seedlings grown in $(\text{NH}_4)_2\text{SO}_4$ is partly, though not necessarily wholly, due to a reduced mobilization of fat.

Acid value of fat. The acid value of the fat in the ungerminated seed was low, but as germination proceeded it increased considerably in the fat of seedlings grown in water and in all the solutions so far tested (Table 1). In those instances where the mobilization of fat was rather more rapid than in seedlings grown in water, e.g. when KNO_3 and sodium



(a)



(b)



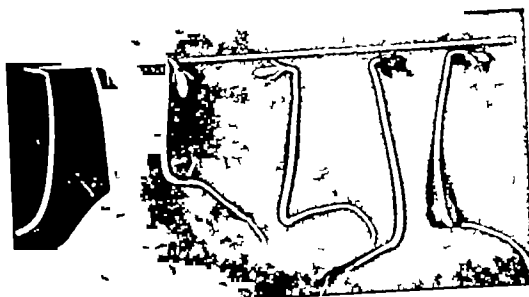
(c)



(d)



(e)



(f)

Seedlings germinated in water (a) (b) seedlings germinated in $(\text{NH}_4)_2\text{SO}_4$ (0.02M) (c) (d) seedlings germinated in $(\text{NH}_4)_2\text{SO}_4$ (0.02M) + sodium succinate (0.02M) (e) (f) Photographs (a) (c) and (e) are pictures of seedlings germinated in jars and (b) (d) and (f) are photographs of separate seedlings to show more clearly the form of growth

succinate were supplied, the acid values were slightly higher. On the other hand, where the mobilization of fat was retarded, as when $(\text{NH}_4)_2\text{SO}_4$ was used, the acid values were slightly lower than when the seedlings were grown in water.

Iodine value of fat The iodine values of the fat of seedlings grown in water, $(\text{NH}_4)_2\text{SO}_4$ and CH_3COOK , are also shown in Table 1. It will be seen that, although the iodine value of the fat at 48 hr had dropped slightly as compared with that in the ungerminated seed, there was no evidence of any significant rise or fall during the later stages of

of their influence on the hexoses or ascorbic acid, because sodium succinate, for example, does not increase the former, and though it increases the latter, this can be done also by other means, e.g. by supplying ascorbic acid in combination with $(\text{NH}_4)_2\text{SO}_4$, without any resultant modification of the form of the hypocotyls.

When mannose was supplied, growth was very poor, and, as in the case of seedlings grown in $(\text{NH}_4)_2\text{SO}_4$, the hypocotyl was bent. Wachtel (1943), also using cress (*cardamine*) seedlings, has found that mannose inhibited growth.

Table 1 *Acid and iodine values of the fats of cress seedlings*

Duration of exp (hr)	Acid values Fat of ungerminated seed, 16						Iodine values Fat of ungerminated seed, 140		
	Exp 1 treatment		Exp 2 treatment		Exp 3 treatment		Exp 4 treatment		
	$(\text{NH}_4)_2\text{SO}_4$ (0.02 M)		KNO_3 (0.02 M)		Sodium succinate (0.02 M)		$(\text{NH}_4)_2\text{SO}_4$ (0.02 M) CH_3COOK (0.02 M)		
	Water		Water		Water		Water		
48	5.9	4.4	11.7	10.3	5.7	5.0	129.1	131.9	132.3
72	8.8	6.2	—	—	8.4	11.1	130.9	128.8	124.2
96	14.6	13.1	22.2	22.3	11.7	14.3	130.0	129.1	129.7
120	20.7	14.0	—	—	17.0	19.2	126.7	128.9	126.2
144	25.9	19.5	36.7	46.0	—	39.2	128.9	—	118.7
168	50.2	32.2	39.1	53.4	47.0	55.0	127.0	133.9	—

growth. This is in contrast to the findings of Ivanow (1912), who observed that in poppy seedlings 8 days old the iodine value of the fat was 71.6, as compared with 140.2 in the fat of the ungerminated seedling. He also obtained similar results with linseed. This rapid fall in iodine value was attributed to the relatively rapid disappearance of the more highly unsaturated fatty acids. Miller (1912), in experiments on the sunflower, found that the iodine value of the fat of the hypocotyls and roots fell very considerably from the fourth to the fourteenth day of growth (from 117.7 to 48.3), whereas the iodine value of the fat from the cotyledons showed much less reduction during this period (from 125.5 to 111.7). The results of these two workers suggest that the unsaturated acids were preferentially utilized for conversion into hexose, but there was no clear evidence of this in our experiments.

Influence of the nutrient medium on the form of growth of cress seedlings

Salts which depressed the synthesis of ascorbic acid, e.g. $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , NH_2OH , HCl , also influenced the form of growth (Pl. 1). Seedlings grown in water had erect and rather elongated hypocotyls, whereas in those grown in $(\text{NH}_4)_2\text{SO}_4$ the hypocotyls were bent, with a hooked appearance. When the sodium salts of succinic, pyruvic or acetic acids were supplied in addition to $(\text{NH}_4)_2\text{SO}_4$, the type of growth much more closely resembled that of seedlings grown in water. The reason for this is not clear. Evidently the salts do not operate by reason

Our results show that a close correlation exists between ascorbic acid and hexose sugars in the seedling, irrespective of whether it is germinated in water or in salt solutions which increase or decrease the synthesis of the vitamin. The simplest assumption is that hexose is converted to ascorbic acid, and the gradients of the curves expressing the relation between these constituents suggest that the conversion may be influenced by the nature of the salt present. On this hypothesis, Na or K salts containing a readily utilizable anion (e.g. HCO_3^- , pyruvate, succinate, etc.) favour the synthesis of ascorbic acid because they increase the conversion of hexose to ascorbic acid. Conversely the reduced synthesis of ascorbic acid in the presence of ammonium salts containing a utilizable anion, and the still poorer synthesis with ammonium salts containing a non-utilizable anion (e.g. SO_4^{2-} or Cl^-) may be explained as being due to a decrease in the efficiency of conversion of hexose to ascorbic acid.

This interpretation of the experimental data is supported by the fact that when glucose is supplied to seedlings growing in any one salt solution, the increase in the ascorbic acid synthesized is quantitatively what would be expected by extrapolation of the curve relating ascorbic acid to hexose formation for that particular salt. Thus glucose supplied to the seedling behaves in the same way as hexoses derived from the seed reserves in relation to the formation of ascorbic acid.

The reason why these various salts should affect the conversion of hexose to ascorbic acid has still to be found. It is significant, however, that a correlation exists between the effect these salts have on the hexose/ascorbic acid relationship and their effect on the pH of the cell sap. Thus salts which induce a more alkaline pH of the cell sap favour the conversion of hexose to ascorbic acid and salts which depress the pH decrease the conversion. So far we have encountered no exception to this rule.

In this discussion we have assumed that the changes in ascorbic acid observed with the different treatments are due principally to an alteration in the synthetic activity of the seedling. It is possible, however, that other factors are operating. The concentration of ascorbic acid in the cell at any time may well be conditioned *inter alia* by two factors: (1) the rate of synthesis of ascorbic acid from a precursor such as hexose sugars, and (2) the rate of oxidation of ascorbic to dehydroascorbic acid. The small amount of ascorbic acid produced by seedlings growing in $(\text{NH}_4)_2\text{SO}_4$ could therefore be the result of either a defective synthetic mechanism or of an increase in the rate of oxidation to dehydroascorbic acid brought about by a general rise in the oxidation potential of the system. The further possibility that both factors may be operative cannot be excluded. Similarly, the large amounts of ascorbic acid found in the presence of certain K or Na salts may well be due to a reversal of these effects. It is hoped to present evidence on this subject in a further communication.

It is of interest that the synthesis of ascorbic acid was greatly reduced when D-mannose was supplied. This is in contrast to the observations of Tadokoro & Nisida (1940), who found that the ascorbic acid content of maize and soya bean grown in a nutrient solution was increased by the addition of mannose. We have already referred to the work of Ray (1934), whose results agree with those of Tadokoro. It should be noted that these two workers were using plants belonging to the Gramineae and Leguminosae, while we used a cruciferous plant.

The inhibitory effect of D-mannose on the synthesis of ascorbic acid was diminished by the presence of D-glucose, D-galactose and D-fructose, sugars which, when supplied alone, favoured the synthesis of ascorbic acid. It is possible, therefore, that we have here an instance of inhibition of the competitive rather than the non-competitive type, that is, the glucose and mannose are in competition for the surface of some enzyme associated with the synthesis

of ascorbic acid. If this is so, then the contrast between the effect of D-glucose and D-galactose and that of D-mannose suggests that the stereochemical configuration of C-3 in sugars is important in determining whether these can be converted into ascorbic acid.

SUMMARY

1. In cress seedlings germinated in the presence of various salt solutions in the dark, a close correlation was generally found between the amount of ascorbic acid and hexose sugars synthesized.

2. Salts, particularly those of ammonium, which contained an anion not readily utilized, reduced both these constituents. Sodium and potassium salts containing an anion readily utilized (e.g. bicarbonate, pyruvate, succinate) increased the ascorbic acid but not the hexoses.

3. Solutions of D-glucose, D-galactose and D-fructose alone or combined with various salt solutions increased the hexose sugars. Ascorbic acid was also increased, to an extent determined by the effect produced on the hexose/ascorbic acid relationship by the individual salts. There was no evidence that the hexose supplied behaves differently from that derived from the seed reserves.

4. It is suggested that hexose is converted into ascorbic acid and that the efficiency of the conversion is influenced by the nature of the salt present, possibly through its effect on the pH of the cell sap. Salts which raise the pH increase, and those which lower it decrease, the efficiency of conversion.

5. The rate of mobilization of fat was slower in seedlings grown in ammonium sulphate than in those grown in water, and may partly account for the lowered formation of sugars in the former.

6. In contrast to the other hexose sugars, D-mannose depressed the synthesis of ascorbic acid, but its action could be diminished by supplying in addition D-glucose, D-fructose or D-galactose.

7. The form of growth of the seedlings was modified in the presence of ammonium sulphate or ammonium chloride, but became more nearly normal when such salts were supplied with a sodium or potassium salt containing a utilizable anion.

We wish to express our thanks to Drs J. Barker and L. J. Harris for their criticism and advice, and to acknowledge the technical assistance of Miss N. L. Gass. The work described in this paper was carried out as part of a programme of the Food Investigation Organization of the Department of Scientific and Industrial Research by arrangement with the Medical Research Council.

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Studies on Metabolic Products of the *Penicillium luteum* Series

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(Received 18 February 1949)

Considerable attention has been devoted to the polysaccharide metabolic products of *Penicillium luteum* Zukal. Raistrick & Rintoul (1931) isolated the mucilaginous polysaccharide, luteic acid, which was shown to be a malonylpolyglucose. Alkaline or mild acidic hydrolysis of luteic acid yielded the acid free polysaccharide, luteose, which was constituted mainly of β glucose units linked through the 1 6 positions (Anderson, Haworth, Raistrick & Stacey, 1939). Luteic acid was elaborated by *P. luteum* when grown on a number of different hexoses, pentoses or glycerol as sole source of carbon (Birkinshaw & Raistrick, 1933). Later, Anderson & Raistrick (1936) showed that although luteic acid was the main metabolic product of *P. luteum* Zukal, other laevorotatory polysaccharides, built up of mannose, galactose and fructose units, were produced in proportions varying with the age of the culture. Anderson *et al* (1939) isolated a laevorotatory polysaccharide which gave D galactose and D mannose on hydrolysis and for which the name galuteose was proposed.

The present work is concerned with the isolation of two hitherto undescribed polysaccharides, the properties and component sugar residues of which have been investigated. A study of the sedimentation of the two new polysaccharides in the ultracentrifuge is presented in the following paper (Ogston, 1949).

EXPERIMENTAL

Strains of Penicillium luteum

Six strains of *P. luteum* were used, they are distinguished by their laboratory catalogue numbers. The strains were broadly similar in their cultural characteristics and morphological appearance and clearly belong to the *P. luteum* series. Strains F 6, 39, 46, 51 and 52 agreed closely with Thom's

(1930) description of *P. luteum* Sopp and F 231 was a conidial non ascospore form closely corresponding to *P. luteum* Zukal. Strains F 6, 39, 46, 51 and 52 give broadly spreading floccose colonies on Czapek Dox agar which were yellow at the margin and slowly developed greyish green conidial areas in the centre. The reverse was orange to red in colour. On beer wort agar, broadly spreading thick floccose felts developed with zonate bands of yellow, pink and green conidial areas, the reverse was orange to red. On nutrient gelatin medium, broadly spreading mainly white floccose, buckled colonies developed with some pink zones and green conidial areas. The gelatin was liquefied and became red in colour. These strains were isolated in the laboratory, F 39 from mouldy cake, F 46 from a solution of sodium carboxymethylcellulose and F 51 and 52 from an industrial acetone recovery tower. Strain F 231 was purchased from the National Collection in January 1947 as *P. luteum* Zukal NCTC 585. On Czapek Dox agar it formed broadly spreading mainly white floccose colonies with marginal green conidial zones, the reverse was colourless. Similar colonies were formed on nutrient gelatin medium, which was liquefied and remained colourless. In all the above strains the sterigmata were acuminate, $10-11 \times 2.5 \mu$ and conidia ovoid about $2.5 \times 3.5 \mu$.

Cultural methods

The cultures were grown in glass culture vessels ('Glaxo bottles') each containing 350 ml of Czapek Dox medium of the following composition: glucose, 50 g; NaNO_3 , 20 g; K_2HPO_4 , 10 g; KCl , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g; distilled water 1 l. After sterilization by autoclaving each vessel was sown with a spore suspension from 7-day old beer wort agar slopes. The cultures were incubated at 25° in darkness, and usually harvested when the glucose was virtually completely exhausted. Initial and final glucose concentrations were determined in samples of the culture filtrates by the Fehling-Sorby method in which cuprous oxide was determined iodometrically by the method of Shaffer & Hartmann (1921). Carbon was determined by the wet combustion method of Houghton (1945). Volatile neutral

compounds, volatile acids and non volatile acids were determined by methods based on those of Birkinshaw & Raistrick (1931). Data based on single flask cultures of the six strains are summarized in Table 1, and show that from 11 to 54% of the glucose carbon fermented by the various strains was converted to soluble metabolic compounds in the filtrates. All the cultures produced acid during growth, but there was no evidence of the formation of significant quantities of volatile acids or volatile neutral compounds.

pH 4.92 and on electrometric titration (glass electrode) 25.0 ml 0.1N NaOH were required to neutralize the filtrate to pH 7.0. Under corresponding conditions, luteic acid (5 g) required 22.5 ml N NaOH for neutralization (Raistrick & Rintoul, 1931). The filtrate had a reducing value (a.v.) corresponding to 6.98 g/l apparent glucose, which was equivalent to 32.8% of the crude polysaccharide (38.7% on an ash free basis). The reducing value before hydrolysis was 3.3% as apparent glucose. The substance gave no reaction

Table 1 *Preliminary analysis of carbon compounds present in culture filtrates of strains of Penicillium luteum*

P luteum Strain F	Time of growth (days)	pH		Carbon (g/l)			Volatile acids (ml N acid/l)	Non volatile acids (g Ca salt/l)	Metabolic carbon compounds (% initial C)	Metabolic carbon compounds (% C fermented)
		Initial	Final	Initial	Final total	Final glucose				
6	12	5.8	3.5	19.12	8.83	0.60	0.11	0.0	43.0	44.4
39	18	5.5	4.7	20.20	5.02	0.90	—	—	20.4	21.4
46	25	7.0	2.9	18.51	10.62	1.24	0.46	4.2	50.7	54.3
51	24	5.5	3.3	20.20	9.78	0.80	0.69	0.0	44.4	46.3
52	18	5.5	—	20.20	2.32	0.0	—	—	11.5	11.5
231	92	5.8	3.5	18.72	11.10	8.12	—	—	15.9	28.1

Isolation of polysaccharides

Preliminary experiments showed that the culture filtrates contained polysaccharides, specimens of which were isolated, as follows. Spores of each of the six strains were seeded into groups of ten flasks, containing 350 ml of medium (initial glucose, 49.0 g/l). At the completion of fermentation (Table 2) the culture filtrates were separated from the mycelial felts by filtration through fluted paper. The filtrates were evaporated *in vacuo* at low temperature to 0.1 vol and the polysaccharides precipitated by addition of 4 vol of ethanol. The polysaccharides separated out as heavy syrupy precipitates which were desiccated with difficulty by titration with increasing strengths of ethanol. They were finally washed with absolute ethanol and ether, and dried *in vacuo* over P₂O₅. The yields and some of the properties of the crude products are summarized in Table 2.

with iodine. A sample of the crude product (15.0 g) in water (30 ml) was dialysed in a collodion sac against six changes of distilled water for 66 hr. The polysaccharide was recovered by concentration *in vacuo* to 30 ml and addition of ethanol to 66% concentration (w/w) obtained 4.39 g (29.3%). The low recovery on dialysis suggested that the polysaccharide may have a comparatively low molecular weight and undergo slow diffusion. The dialysed product had an ash content of 2.2%.

Tollens's phloroglucinol test was negative indicating the absence of pentose residues. The naphthoresorcinol reaction for uronic acids was negative. A negative result was also obtained with Dische's (1947) carbazole reaction for uronic acids. The crude polysaccharide gave a positive reaction with the Selwanoff resorcinol reagent, indicating the presence of ketose sugars.

The polysaccharides from the other strains in 100 ml aqueous solution, adjusted to pH 2 with HCl, were freed from

Table 2 *Isolation and properties of crude polysaccharides from strains of Penicillium luteum*

Strain F	Time of incubation (days)	Culture filtrate		Crude polysaccharide				Oxidation product	
		Final glucose (g/l)	pH	Yield (g)	Yield (% of initial glucose)	Ash (%)	$[\alpha]_D^{25}$ (°)	Substance and m.p.	Yield (% of polysaccharide)
6	21	1.0	3.93	18.10	10.6	15.2	+ 7.0	Mucic acid, 206°	22.8
39	21	1.4	3.11	34.43	20.1	13.8	+ 5.8	Mucic acid, 214°	17.9
46	21	1.3	2.97	30.68	17.9	11.7	+ 8.5	Mucic acid, 209–211°	12.6
51	22	0.6	3.02	41.64	24.3	10.3	+ 5.7	Mucic acid, 215°	11.0
52	22	1.6	3.10	18.54	10.8	15.0	0.0	Mucic acid absent	—
231	41	2.0	3.88	27.77	16.2	19.6	+ 12.9	Mucic acid absent	—

The properties of the crude polysaccharide from strain F 6 were examined in some detail. On ignition the substance gave 15.2% of colourless ash. Its specific rotation, $[\alpha]_D^{18}$, was +7° in water (c, 1), crude luteic acid has $[\alpha]_D$ -25 to -30°. A sample of the polysaccharide (5.32 g) was hydrolysed with N H₂SO₄ (50 ml) at 100° for 7.5 hr. An equivalent of saturated Ba(OH)₂ solution was added to the cooled hydrolysate, the mixture diluted to 250 ml and filtered. The filtrate gave

ash and low molecular impurities by dialysis in collodion sacs against repeated changes of distilled water until the dialysate was neutral (72 hr). The dialysed polysaccharide solutions were evaporated *in vacuo* to about 10% concentration and fractionated by addition of increasing proportions of ethanol. Properties of the isolated fractions are summarized in Table 3. Acid equivalents were determined by titration of 0.02 g of polysaccharide in 5 ml water with 0.01N NaOH (CO₂ free)

Table 3 *Ethanol fractionation of crude polysaccharides from strains of Penicillium luteum*

Source of polysaccharide, etc	Fraction no	Conc of ethanol (% w/w)	Weight (g)	Acid equiv	$[\alpha]_D^{20}$ (°)
Strain F 6 (25.02 g dialysed, recovery, 14.50 g, 57.9%)	(Insol.)	0	1.28	—	—
	1	44.1	4.09	4730	—
	2	61.2	4.10	6060	+21.6
	3	70.3	2.62	530	-18.1
	4	76.0	1.06	450	-14.0
Strain F 39 (39.63 g dialysed, recovery, 4.82 g, 12.2%)	(Sol.)	—	1.35	—	—
	1	50.8	2.30	1930	+5.0
	2	56.5	1.78	2670	+11.0
	3	63.0	0.38	1240	-7.2
	4	70.3	0.17	890	—
Strain F 46 (38.2 g dialysed, recovery, 11.36 g, 29.7%)	(Sol.)	—	0.04	—	—
	(Insol.)	0	0.15	—	—
	1	39.3	0.90	—	—
	2	48.5	0.12	1260	—
	3	53.5	0.35	1790	+0.9
Strain F 51 (35.6 g dialysed, recovery, 6.50 g, 18.4%)	4	54.8	0.73	2380	+14.4
	5	56.9	1.06	2850	+16.3
	6	59.8	0.45	730	-13.8
	7	69.1	0.08	1300	+1.3
	8	70.0	2.85	630	-14.8
Strain F 52 (41.7 g dialysed, recovery, 15.92 g, 38.2%)	9	70.5	1.78	620	-19.2
	10	76.0	0.05	—	—
	(Sol.)	—	0.43	—	—
	1	47.0	4.32	1440	-2.0
	2	58.9	1.91	1180	-13.0
Strain F 231 (19.0 g dialysed, recovery, 1.68 g, 8.8%)	3	63.4	0.09	—	—
	4	76.0	0.05	—	—
	(Sol.)	—	0.10	—	—
	1	54.5	8.99	2060	+17.6
	2	58.0	2.53	600	-12.0
Strain F 231 (19.0 g dialysed, recovery, 1.68 g, 8.8%)	3	63.0	2.60	440	-22.5
	4	70.2	1.20	440	-22.2
	5	76.0	0.21	440	-22.3
	(Sol.)	—	0.39	—	—
	1	52.0	0.17	3320	—
Strain F 231 (19.0 g dialysed, recovery, 1.68 g, 8.8%)	2	56.0	0.55	13000	+21.7
	3	65.7	0.64	6210	+13.9
	4	71.8	0.12	5470	—
	5	76.0	0.05	—	—
	(Sol.)	—	0.15	—	—

Table 4 *Properties of polysaccharide fractions (Strain F 6)*

Fraction	$[\alpha]_D^{20}$ (°)	Ash (%)	pH of 1% solution	R V (% apparent glucose)		Mucic acid on oxidation (%)
				Before hydrolysis	After hydrolysis	
1	—	0.55	4.21	1.50	86.0	38.3 (m p 210°)
2	+21.6	0.20	4.91	1.49	92.8	—
3	-18.1	0.0	3.20	3.81	84.3	3.3 (m p 210°)
4	-14.0	0.80	3.46	8.73	79.7	—

using phenolphthalein as indicator. Specific rotation data refer to aqueous solutions, $c=1$. In the case of the fractions from strain F 6, reducing power was determined before and after hydrolysis and the products of nitric acid oxidation examined (Table 4). Reducing power before hydrolysis was determined with 0.02 g quantities by Hanes's modification of the method of Somogyi (1937 see Hockenfull & Herbert, 1945). For determination of reducing power after hydrolysis the polysaccharides (0.02 g) were hydrolysed for 2 hr at 100° with 2N HCl in sealed tubes, neutralized, and reducing

sugars determined as above. Samples of polysaccharide (0.5 g) were oxidized by the method of Kent & Tollens (1885), and the yield of mucic acid and its melting point recorded (All melting points are corrected.)

Strain F 6 was shown to yield a mixture of at least two polysaccharides which were separated by ethanol fractionation into a dextrorotatory, neutral polysaccharide (F 1 and F 2) and a laevorotatory, acidic polysaccharide (F 3 and F 4). The former appeared to predominate in the crude polysaccharide. The dextrorotatory polysaccharide yielded mucic

acid (38.3%) on nitric acid oxidation, whereas the laevorotatory polysaccharide gave only an insignificant yield of the acid

Strain F 39 gave a dextrorotatory, neutral polysaccharide, and in addition a very small amount of a laevorotatory polysaccharide

Strain F 46 crude polysaccharide was shown to contain a dextrorotatory, neutral polysaccharide and a larger quantity of a laevorotatory, acidic polysaccharide

Strain F 51 gave laevorotatory products only, which were weakly acidic (acid equivalent 1200)

Strain F 52 gave approximately equal quantities of a dextrorotatory, neutral polysaccharide and a laevorotatory, acidic polysaccharide ($[\alpha]_D^{20} -22^\circ$, acid equivalent 440 corresponding to approximately one equivalent of acid for every three anhydrohexose units in the polysaccharide) Fraction 1 (7.8 g) was refractionated between ethanolic concentrations of 50 and 60% (w/w) and shown to consist mainly of a dextrorotatory, neutral polysaccharide ($[\alpha]_D^{16} +21.6$, acid equivalent 3000, 4.27 g) Nitric acid oxidation of fraction 3 ($[\alpha]_D -22.5^\circ$) gave oxalic acid (6.3%), and no mucic acid was detected

Strain F 231 crude polysaccharide gave a very low recovery (8.8%) on dialysis The product consisted of dextrorotatory, neutral polysaccharides No laevorotatory substances were detected Nitric acid oxidation of fraction 2 gave mucic acid (m.p. 207°, yield 43.9%) This fraction had n_D before hydrolysis of 0.5% as apparent glucose It corresponded closely in its properties to the dextrorotatory polysaccharide isolated from strain F 6

Dextrorotatory polysaccharide from strain F 6

Isolation and properties of the polysaccharide Preliminary experiments showed that the most satisfactory method of isolation of the pure dextro and laevorotatory polysaccharides was by further ethanolic fractionation of the dextro and laevorotatory fractions obtained in the preliminary ethanolic fractionation of the dialysed polysaccharide The dextrorotatory polysaccharide was normally precipitated when the ethanolic concentration reached 50–60% The following results were obtained in a typical experiment The crude polysaccharide (40.7 g) was dissolved in water and the insoluble fraction centrifuged off The latter was twice extracted with water and the extracts combined with the main extract The dried insoluble fraction weighed 3.57 g

The solution (150 ml) was made acid to pH 2 with HCl and dialysed for 6 days against several changes of distilled water The dialysed polysaccharide solution was concentrated *in vacuo* to 63 g and ethanol added to 50% concentration (w/w) The precipitated polysaccharide was dried in the usual way, 8.18 g, acid equivalent 6000 The polysaccharide (6.93 g) was dissolved in water (100 ml) and divided into seven fractions by addition of increasing proportions of ethanol (Table 5) The acid equivalents and specific rotations of the fractions were determined The main fractions (nos 3–5, 5.25 g, 75.7% of the starting material) were practically identical, being virtually neutral and having specific rotations in the range $+23.0$ to $+29.7^\circ$ Further ethanolic fractionation showed that fraction 3 consisted essentially of polysaccharide with specific rotation of $+27.5^\circ$ Fractions with specific rotations in the range $+27.5$ to $+29.7^\circ$ were regarded as the pure dextrorotatory polysaccharide and were used in the experiments described below The pure substance was ash free, it contained 0.2–0.5% N, but was free from S and halogens

Hydrolysis of polysaccharide Preliminary experiments showed that liberation of reducing sugars from the polysaccharide on hydrolysis with $2N H_2SO_4$ at 100° was at a maximum after 8 hr The polysaccharide (1.93 g) in $2N H_2SO_4$ (100 ml) was hydrolysed under these conditions The ice cold hydrolysate was carefully neutralized with hot saturated $Ba(OH)_2$, a few drops of $N H_2SO_4$ added and finally neutralized with an excess of pure $BaCO_3$ The precipitated $BaSO_4$ was centrifuged off and washed, the combined supernatant solution and washings were evaporated *in vacuo* to 100 ml The neutralized hydrolysate had a total reducing value corresponding to 1.50 g apparent glucose, i.e. 77.8% of the polysaccharide (1.56 g as apparent galactose, i.e. 80.9% of the polysaccharide), its optical rotation α_D^{16} was $+1.065^\circ$ (1 dm) and specific rotation $[\alpha]_D^{16} +71.0^\circ$ (n_D as glucose) and $+68.2^\circ$ (n_D as galactose)

Identification of component sugars Partition chromatograms on paper were made with samples of the neutralized hydrolysate described above (Partridge, 1946, 1948, Forsyth, 1948) The chromatograms were developed for 72 hr at 20° n -Butanol, ethanol, water (40, 10, 50% v/v) and n -butanol were used as solvents D -Galactose, D -glucose, D -fructose and D -mannose were included as reference sugars

Butanol-ethanol-water The chromatograms gave only a single spot corresponding to galactose Glucose and mannose

Table 5 *Homogeneity of dextrorotatory polysaccharide (Strain F 6)*

(Dextrorotatory fractions of dialysed polysaccharide were refractionated with ethanol and properties of the fractions determined)

Fraction	Conc of ethanol (%, w/w)	Weight (g)	Acid equiv	$[\alpha]_D^{20}$ ($^\circ$)	Nitrogen (%)
1	20.5	0.20	3100	—	—
2	49.3	0.40	6300	—	—
3	52.5	2.44	15000	$+23.0$	—
3a*	—	—	10000	$+27.5$	0.47
4	54.8	1.95	14000	$+28.9$	—
5	57.7	0.86	24000	$+29.7$	0.18
6	69.0	0.26	7000	—	—
7	Mother liquor	0.23	940	—	—
Recovery, 6.34 g, 91.5%					

* Fraction 3a was obtained by reprecipitation of fraction 3, three times between 49.5 and 53.0% alcoholic concentration

were absent. The sensitive resorcinol reagent (Forsyth, 1948) was used to demonstrate the absence of ketose sugars.

Butanol The chromatograms clearly showed the presence of galactose and the absence of glucose, mannose and fructose.

Quantitative determinations of galactose in the polysaccharide hydrolysate were carried out by the partition chromatographic technique described by Flood, Hirst & Jones (1947, 1948). The polysaccharide (10 mg) and L-rhamnose (5 mg) as reference sugar were hydrolysed with 0.4 ml 2 N H_2SO_4 in a sealed tube for 1 hr at 100°. The neutralized hydrolysate was transferred to the chromatograms and developed with *n*-butanol, ethanol, water (40, 10, 50% v/v) for 72 hr at 20°. Recoveries of galactose and rhamnose corresponding to 88% of the former in terms of the polysaccharide were obtained.

Yeast fermentation of polysaccharide hydrolysate The chromatographic analyses described above showed that galactose was the main component sugar of the polysaccharide. The chromatograms demonstrated the absence of significant quantities of glucose, fructose and mannose. The absence of reducing sugars, other than galactose and those normally fermented by yeast, was confirmed by fermentation of the neutralized hydrolysate with a strain of *Saccharomyces carlsbergensis* Hansen (NCTC 742), which had been trained to ferment galactose. The organism was passaged three times in a medium containing 10 parts of hydrolysed lactose (5 g lactose/l) hydrolysed with 0.5 N HCl for 1.5 hr at 100° and neutralized to pH 5-6 and 1 part of beer wort. The cells were washed twice with saline and suspended in the polysaccharide hydrolysate at 30° and pH 5-6 so that the cell density was about 80×10^4 /ml and fermentation was allowed to proceed for 96 hr (Wise & Appling, 1944). A parallel fermentation with a similar concentration of D-galactose was carried out as a control. Reducing value determinations were made with 0.2 ml samples of the supernatant solutions, freed from cells by centrifugation, by Hanes's modification of Somogyi's (1937, see Hockenfull & Herbert, 1945) method (Table 6). The

acetic acid (5 ml) added to give an acetic acid concentration of about 90% and the mixture allowed to stand at 0°. After seeding with a trace of D-galactose a mass of colourless crystals was obtained which was filtered off and washed with glacial acetic acid, yield of crude D-galactose, 1.24 g (51.0%). The crude product (m.p. 160-163°) was once recrystallized from 90% acetic acid, and almost pure D-galactose obtained, m.p. 163°, unchanged on admixture with authentic D-galactose, m.p. 166° $[\alpha]_D^{18} + 113^\circ$ after 5 min $\rightarrow +70^\circ$ after 19 hr.

D-Galactose methylphenylhydrazone On treatment with 0.2 g methylphenylhydrazine (asym. redistilled b.p. 104° at 14 mm) and 0.1 g 50% acetic acid the sugar (0.25 g) in 50% ethanol gave crystalline D-galactose methylphenylhydrazone (0.33 g, 84% of the theoretical, Neuberg, 1907, Lüdtke, 1929). The crude product was recrystallized from hot pyridine, ethanol (1:1, v/v) mixture, after one to two crystallizations pure D-galactose methylphenylhydrazone, m.p. 189°, was obtained. The melting point was undepressed on admixture with the authentic hydrazone (m.p. 189°) (Found (Weiler and Strauss) C, 54.8, H, 7.1, N, 9.2. Calc for $C_{12}H_{20}N_2O_5$, C, 54.9, H, 7.1, N, 9.0%).

A further galactose determination on the neutralized hydrolysate was made by the methylphenylhydrazone method of Hirst, Jones & Woods (1947). A portion of the hydrolysate corresponding to 0.9637 g of polysaccharide was treated with the methylphenylhydrazine reagent and gave 0.7707 g galactose methylphenylhydrazone, which corresponds to 0.5358 g galactose according to the formula of Hirst *et al.* (1947), i.e. to 55.6% of the polysaccharide.

The excess methylphenylhydrazine was removed as benzaldehyde methylphenylhydrazone from the mother liquor in the above determination as described by Morgan (1938). The volume of the residual solution was adjusted to 25 ml. The total reducing value corresponded to 0.0578 g apparent glucose, i.e. 6.0% of the polysaccharide. The remainder (24 ml) was evaporated to a syrup, dissolved in methanol (5 ml.) and treated with *p*-nitrophenylhydrazine (0.1 g) as described by van Ekenstein & Blanksma (1903, Reclaire, 1903). No crystalline hydrazone was obtained under these conditions.

D-Galactose *p*-nitrophenylhydrazone Neutralized hydrolysate equivalent to 2.275 g polysaccharide was evaporated *in vacuo* to a thick syrup, which was dissolved in methanol (15 ml.) and heated with *p*-nitrophenylhydrazine (2.0 g) for 10 min. on the water bath. A bright yellow microcrystalline solid soon separated on cooling. The product was filtered off on a sintered glass crucible and washed with ice-cold ethanol and ether. On evaporation of the mother liquor and washings nearly to dryness *in vacuo* and addition of absolute methanol (10 ml.) further crops of the hydrazone were obtained. Yield of crude hydrazone 2.255 g, corresponding to 56.6% of the polysaccharide. The crude substance softened at 184° and melted with decomposition at 185°. It was recrystallized from boiling absolute ethanol, m.p. 194°. On admixture with authentic D-glucose *p*-nitrophenylhydrazone (m.p. 186°) the melting point was depressed to 184°. On admixture with authentic D-galactose *p*-nitrophenylhydrazone (m.p. 195°) the melting point was undepressed at 194° (Found C, 46.0, H, 5.3, N, 12.7. Calc for $C_{12}H_{17}N_3O_7$, C, 45.7, H, 5.4, N, 13.3%).

Acetylation of polysaccharide. Preliminary experiments were made with the crude polysaccharide to determine the optimal conditions for acetylation. The pure substance (0.545 g) was dissolved in water (2 ml.), pyridine, redistilled

Table 6. Fermentation of dextrorotatory polysaccharide hydrolysate by *Saccharomyces carlsbergensis*

Time (hr)	Polysaccharide hydrolysate			D-Galactose		
	Reducing value of fermentation mixture as apparent glucose (mg/100 ml)	Residual glucose x v (%) of initial		Reducing value of fermentation mixture as apparent glucose (mg/100 ml)	Residual glucose x v (%) of initial	
0	383	100		243	100	
72	95	25		65	27	
96	84	22		23	09	

galactose was rapidly fermented until less than 1% of the original reducing power remained after 96 hr. Similar results were obtained with the hydrolysate, which retained only 2.2% of the original reducing power after 96 hr fermentation. It is concluded that less than 2.2% of the reducing substances (i.e. 1.8% of the polysaccharide) were present as sugars other than glucose, fructose, mannose and galactose.

Isolation of D-galactose and derivatives A sample of neutralized hydrolysate corresponding to 2.435 g polysaccharide was evaporated *in vacuo* to a thick syrup, glacial

over KOH, b p 115° (25 ml), was added and followed by redistilled acetic anhydride (25 ml), the latter was added gradually so that the temperature of the mixture did not exceed 35°. The mixture was kept at 20° for 48 hr, at this stage a sample gave no precipitate on pouring into ice cold water. The solution was heated under reflux at 90° for 6 hr, cooled, and poured into 500 ml of ice cold water giving an immediate precipitate of almost colourless acetyl polysaccharide. The latter was filtered off, washed free from acid with water and dried *in vacuo* over P_2O_5 . Yield, 0.690 g, i.e. 71.2% of the theoretical yield of triacetyl compound. Fractionation of the acetyl derivative showed that it was homogeneous. The crude substance (0.531 g) was dissolved in $CHCl_3$ (15 ml) and three times reprecipitated by addition of 3 vol ether. The third precipitate was redissolved in $CHCl_3$ (10 ml) and fractionated by addition of increasing proportions of ether. The main fractions were precipitated by 59% (v/v) and 75% (v/v) of ether, respectively, their weights were 0.213 and 0.120 g, total recovery 0.405 g (76.3%). The specific rotations of the fractions were respectively, $[\alpha]_D^{17} +38.3^\circ$ and $+44.1^\circ$ in $CHCl_3$ (c, 0.4) and their acetyl contents, 46.2 and 49.2% (theory for triacetyl hexosan, 44.8%).

Benzoylation of polysaccharide The polysaccharide (1.04 g) was benzoylated by the Schotten Baumann method. The crude benzoylated product (1.66 g, 54.5% of the theoretical for the tribenzoyl hexosan) separated almost immediately as an almost colourless precipitate. It was soluble in acetone and $CHCl_3$, but insoluble in ether and water. After fractional precipitation from $CHCl_3$ solution with ether the main fraction had a specific rotation of $[\alpha]_D^{18} +18^\circ$ in $CHCl_3$ (c, 0.6).

Laevorotatory polysaccharide from strain F 6

Isolation and properties of the polysaccharide After removal of the dextrorotatory polysaccharide from solutions of the crude dialysed polysaccharide by addition of ethanol to 50–60% concentration, the mother liquor consisted predominantly of laevorotatory polysaccharide. A portion of crude laevorotatory polysaccharide precipitated from these mother liquors by increasing the ethanolic concentration to 80% (w/w) (9.68 g, $[\alpha]_D^{16} -2.1^\circ$, acid equivalent 770) was redissolved in water (200 ml), centrifuged to remove insoluble material and the clear, pale golden brown coloured supernatant solution divided into six fractions by addition of increasing concentrations of ethanol. (The insoluble fraction formed a thick viscous paste on addition of water, but did not dissolve. It was readily soluble in $N NaOH$ and slowly soluble in $2N Na_2CO_3$, but insoluble in acids. No precipitate was obtained on acidification of the alkaline solution.) After removal of the first ethanolic precipitate (61.5% ethanol), the supernatant solution was decolorized by treatment with animal charcoal (2 g) at 20° for 18 hr. The charcoal was centrifuged off and the ethanolic fractionation continued. After precipitation and centrifugation of fraction 3, the opalescent mother liquor was evaporated *in vacuo* to 35 g and fractionation continued by addition of sufficient ethanol (70 ml, 61.4%) to produce an appreciable further precipitate. The fractions were precipitated as clear, colourless syrups which were desiccated with difficulty by treatment with increasing concentrations of ethanol, they were finally washed with ether (dried over Na) and dried *in vacuo* over

P_2O_5 . The yields and properties of the fractions are summarized in Table 7. The results showed that the main laevorotatory fractions were practically identical, having specific rotations in the range -13° to -17° and acid equivalents of 680–825 (mean 765, which corresponds to approximately one acidic group per five anhydrohexose units).

Table 7 *Homogeneity of laevorotatory polysaccharide (Strain F 6)*

(Laevorotatory fractions of dialysed polysaccharide were refractionated with ethanol and properties of the fractions determined)

Fraction no	Conc of ethanol (% w/w)	Weight (g)	Acid equiv	$[\alpha]_D^{18}$ (°)
Insol	0	1.294	941	-5.0 (in 0.1N NaOH)
1	51.5	0.183	1580	—
2	59.0	2.713	1296	+5.8
3	68.2	2.962	793	-10.4
3a*	—	—	825	-13.3
4	61.4	0.421	682	-16.9
5	70.3	0.425	790	-14.5
6	75.9	0.038	971	—
Mother liquor	—	0.392	—	—
		Recovery, 8.478 g, 87.5%		

* Fraction 3a was obtained by fractionation of fraction 3, between 75 and 99% ethanol concentration (w/w).

The polysaccharide (10 mg) gave no reaction for ketose sugars in the Seliwanoff test after 15 min at 100°, under the same conditions fructose (2 mg) gave a definite pink coloration after 2–5 min. On hydrolysis with $N H_2SO_4$ (0.0577 g polysaccharide in 25 ml) at 100°, the initial reducing value of 5.3% as apparent glucose rapidly increased to a maximum of 67.8% as apparent glucose after 3 hr, and thereafter decreased at an approximately linear rate, reaching 60.0% after 6 hr. A sample of polysaccharide (0.4984 g) was dissolved in 100 ml $N H_2SO_4$ and hydrolysed under these conditions. The hydrolysate (5 ml) was treated with the equivalent of $Ba(OH)_2$ (12.9 ml, 0.387N) and the precipitate of $BaSO_4$ centrifuged off. The supernatant solution (10 ml) had pH 8.36 and required less than 0.1 ml 0.1N H_2SO_4 for neutralization. It was clear that no acid was liberated on hydrolysis. The remainder of the hydrolysate (≈ 0.440 g polysaccharide) was neutralized in the usual way and the neutralized hydrolysate diluted to 50 ml. Its reducing power was 69.6% (as apparent glucose) and its specific rotation on this basis was $+47.8^\circ$. On heating at 100° for 30 min with phenylhydrazine HCl and sodium acetate, glucosazone was obtained. The solution of neutralized hydrolysate was used in the experiments described below.

Identification of component sugars Chromatographic analyses of the laevorotatory polysaccharide hydrolysate were carried out as described above. The position of the isolated sugars on the chromatogram was detected by means of the ammoniacal $AgNO_3$ and resorcinol reagents.

Butanol ethanol water The chromatograms gave only a single spot corresponding to glucose. Galactose, mannose and fructose were shown to be absent by use of the authentic sugars for reference purposes.

Quantitative determinations of glucose were made by the method of Flood *et al* (1947, 1948). The polysaccharide (11.2 mg) and L-rhamnose (5.0 mg) as reference sugar were hydrolysed with 0.4 ml 2*N* H₂SO₄ in a sealed tube for 1 hr at 100°. The chromatograms were developed with *n*-butanol, ethanol, water (40, 10, 50 v/v) for 72 hr at 20°. The recoveries of glucose and rhamnose corresponded to 63.6% of glucose in the polysaccharide.

Acetylation of polysaccharide. The laevorotatory polysaccharide (0.529 g) was dissolved in water (2 ml) and pyridine (25 ml), and acetic anhydride (25 ml) added as described above. After 48 hr at 20° a colourless precipitate had formed. This was filtered off, washed free from acid with water and dried *in vacuo* (0.588 g, i.e. 62.5% of the theoretical yield of triacetylpolysaccharide). The product had the unusual property of insolubility in CHCl₃, pyridine and acetone as well as in ether and water. (Found C, 47.7, H, 5.7, acetyl, 30.7. C₁₈H₁₁O₈ (C₆H₇O₂(CH₃CO)₃) requires C, 50.0, H, 5.6, acetyl, 44.8%.)

The mother liquor was heated at 90° for 6 hr and poured into ice cold water. No further precipitate of acetylpolysaccharide was obtained.

Benzoylation of polysaccharide. The polysaccharide (0.55 g) benzoylated normally by the Schotten-Baumann method giving 0.96 g (59.6% of the theoretical yield) of crude benzoyl polysaccharide. The crude product (0.793 g) was dissolved in CHCl₃ (5 ml), twice reprecipitated by addition of 3 vol. ether, and redissolved in CHCl₃. The solution was fractionally precipitated with ether and shown to be homogeneous. The pure benzoyl polysaccharide had a specific rotation, $[\alpha]_D^{25} + 3.3^\circ$ in CHCl₃ (c. 0.6).

Action of enzymes on the polysaccharides. Solutions of the dextro and laevo rotatory polysaccharides (2%, 1 ml.) were diluted with water (18.5 ml.) and a 1% solution of a malt amylase (mixture of α and β amylases) preparation (0.5 ml.) added, pH 4.6 and 30°. There was no increase of reducing power after 24 hr incubation. The polysaccharides (2%, 1 ml.) and invertase (yeast autolysate preparation) (0.1 ml.) was added, pH 4.6 and 30°. There was no increase of reducing power after 19 hr incubation. The polysaccharides (2%, 1 ml.) were diluted with water (18.5 ml.) and 1% emulsin (British Drug Houses Ltd., 0.5 ml) added, pH 7 and 30°. There was no change in reducing power after 42 hr incubation. The experiments showed that the polysaccharides were not attacked by α or β amylases, invertase or β glucosidase.

Growth and polysaccharide production by Penicillium luteum F 6 on various carbon sources

A series of media was prepared, based on the Czapek-Dox formula, each containing one of the following substances as carbon source: glucose, galactose, lactose, maltose, soluble starch (2%), glycerol, sodium citrate cryst (9.7%), sodium tartrate cryst (6.4%), sodium acetate cryst (3.8%), sucrose, xylose (1%), dulcitol (1%), mannitol (1%), sorbitol (1%), ethanol (2.5%) (concentration 5%, unless otherwise stated.) Three 'Glaxo' bottle cultures (350 ml. each) were sown with equal volumes of a spore suspension of *P. luteum* F 6 for each carbon source. The initial pH of the media was pH 6-7. Observations on the growth rate of the fungus, the fall of reducing power in the sugar containing media and pH changes are recorded in Table 8. The cultures were harvested after incubation at 25° for the periods stated in

Table 8, the mycelium filtered off, dried and weighed. The culture filtrates were evaporated *in vacuo* to about 0.1 vol and the ethanol insoluble fraction, including polysaccharides, precipitated by addition of a slight excess (2-4 vol.) of ethanol. Samples (0.5 g) of the crude 'polysaccharides' were oxidized with nitric acid by the Kent & Tollens (1885) method, the yields and melting points of the crude oxidation products are recorded in Table 8.

The mycelial growth on the galactose medium was heavier and more vigorous than that on any other medium, it differed significantly from that of the glucose medium, which was white and slimy with a light cream coloured culture filtrate, in its production of green conidial areas. The mycelium was reddish brown on its underside and the culture filtrate reddish brown in colour. Lactose also formed early (5 days) sporulation, but mycelial growth was slow and sparse. No growth took place on citrate or tartrate as sole source of carbon. The acetate medium gave rise to a trace of colourless submerged mycelium. Similar results were obtained with dulcitol, mannitol and sorbitol. Dulcitol and mannitol were recovered unchanged in yields of 95 and 85%, respectively, after incubation for 34 days. A thick syrupy product, which did not crystallize on standing, was obtained from the sorbitol medium, it was probably crude sorbitol. Moderately heavy growth of pink coloured mycelium was obtained on the ethanol medium, which favoured the production of isolated colonies rather than a continuous felt. The observations of mycelial growth, recorded in columns 3-5 of Table 8, were visual estimates made during growth, they were not in every case supported by the mycelial weights finally obtained.

Reducing power determinations after 19 days' incubation showed that galactose was metabolized more rapidly than glucose, at this stage the mean galactose content was 0.60 g/100 ml and that of glucose 0.89 g/100 ml. After incubation for 19 days the pH of the glucose medium fell to 2.63 and ultimately rose again to 3.90, the corresponding values for the galactose medium were 3.96 and 5.14.

The ethanol precipitated fractions from the glucose, galactose, maltose, starch, glycerol, xylose and ethanol media were undoubtedly constituted mainly of polysaccharides. The product from the lactose medium (10.8 g, 20.6%) was the unchanged sugar, it reduced Fehling solution and its m.p. of 200° was undepressed on admixture with authentic lactose. On heating with acetic anhydride and sodium acetate it gave 59% of the theoretical yield of lactose octa-acetate, m.p. 95-100°, undepressed on admixture with authentic lactose octa-acetate. The sparse growth with this disaccharide, as compared with that on media containing its hydrolysis products, suggests that the fungus may not produce lactase. The bulk of the substrate was, however, probably metabolized by some other route since only 21% was recovered and the rotation ($[\alpha]_D^{25} + 1.39^\circ$, 1 dm) of the culture filtrate was not in agreement with its reducing power (4.11 g/100 ml. as apparent lactose) on the assumption that the sugar was the main component. On addition of ethanol (2 vol.) to the concentrated culture filtrate a small immediate precipitate was obtained. On standing for a few hours the supernatant solution deposited lactose as a crystalline mass.

The culture filtrate from the galactose medium was deep red in colour. The coloured component was separated by extraction with CHCl₃ and on evaporation of the solvent orange red crystalline needles were obtained (0.02 g, 0.04% of sugar fermented, m.p. 99-104°).

Table 8 *Growth of Penicillium luteum F 6 on various sources of carbon*

Carbon source	Conc (%)	Mycelial growth after				Sugar conc (% of final culture filtrate)	pH of culture filtrate	Cultures harvested after incubation (days)	Mycelium weight (g)	Yield of poly saccharide		Oxidation product		Reducing value as glucose	
		9 days	21 days	27 days	27 days					(g)	Percentage of substrate	Yield of poly saccharide (%)	Before hydrolysis	After hydrolysis with 2N HCl in sealed tube, 2 hr	
Glucose	5.0	3	4	4	0.19*	3.90	27	6.10	3.64	6.93	Mucic acid, m p 202°	10.9	1.7	58.9	
Galactose	5.0	4	4	4	0.15†	5.14	30	8.70	1.86	3.54	Mucic acid, m p 215°	23.3	1.7	28.9	
Lactose	5.0	1	2	2	4.11†	7.13	30	1.04	Trace	—	—	—	55.7 (83.4§)	82.3	
Maltose	5.0	3	4	4	0.34†	4.00	27	7.38	2.58	4.91	Mucic acid, m p 209°	20.0	2.1	50.3	
Soluble starch	2.0	4	4	4	0.0	7.54	28	3.34	3.64	17.3	Mixture of mucic acid, m p 207° and oxalic acid, m p 99°	2.0	3.0	24.6	
Glycerol	5.0	2	4	4	0.0	5.90	27	0.87	1.45	2.76	Mucic acid, m p 206°	7.6	1.1	26.2	
Citrate (Na ₂ C ₆ H ₅ O ₇ · 5H ₂ O)	9.7	0	0	0	—	—	27	0.0	0.0	0.0	—	—	—	—	
Tartrate (Na ₂ C ₄ H ₄ O ₆ · 2H ₂ O)	6.4	0	0	0	—	—	27	0.0	0.0	0.0	—	—	—	—	
Acetate (NaC ₂ H ₃ O ₂ · 3H ₂ O)	3.8	1	1	1	—	9.13	28	Trace	(2.01)	(5.04)	No mucic acid formed	—	0.7	2.9	
Sucrose	5.0†	2	2	3	2.85†	3.22	34	2.50	1.58	3.01	Oxalic acid, m p 102°	13.3	1.0	48.7	
Xylose	1.0	2	3	4	0.04*	3.84	27	2.55	1.41	13.4	Undetermined	14.7	1.7	14.9	
Dulcitol	1.0	1	1	1	—	7.40	34	0.45	0.0	0.0	—	—	—	—	
Mannitol	1.0	1	3	3	0.0	6.00	34	0.50	0.0	0.0	—	—	—	—	
Sorbitol	1.0	1	1	1	—	7.08	34	0.60	0.0	0.0	Undetermined	—	—	—	
Ethanol	2.5	2	2	3	0.0	5.66	34	1.11	1.47	5.60	Mucic acid, m p 196°	5.5	0.8	15.0	

Mycelial growth key no growth, 0, trace of growth, 1, fairly good growth, 2, good growth, 3, abundant growth, 4
 * As glucose † As sugar specified. ‡ As invert sugar § As anhydrous lactose

After incubation for 28 days the soluble starch medium gave no I_2 reaction, showing that *P. luteum* excretes α amylase into the medium. After concentration to 0.05 vol the culture filtrate was non viscous. The yield of polysaccharide (17.3%) and presence of mucic acid in its oxidation products showed that the starch had been metabolized by the fungus.

The culture filtrate from the acetate medium (pH 9.13) required 10 ml N HCl for neutralization to pH 6.93. The filtrate had no detectable rotation in a 1 dm tube. The ethanolic precipitated fraction was non polysaccharide in character, it gave a negative Molisch reaction and did not reduce Fehling's solution.

Growth and fermentation of the sucrose medium was poor as compared with the media containing the monosaccharides and maltose. After 34 days, 2.85 g/100 ml., i.e. 57% of the initial sucrose, remained unfermented.

Isolation of mucic acid (melting point undepressed on admixture with authentic mucic acid) on oxidation of the polysaccharide fractions from the glucose, galactose, maltose, starch, glycerol, and ethanol media showed that these substrates as sole sources of carbon were metabolized by *P. luteum* with the production of galactose containing polysaccharides.

Growth of Penicillium luteum F 6 on the dextro and laevo rotatory polysaccharides as sole sources of carbon

Dextrorotatory polysaccharide. A medium based on the Czapek Dox formula, containing the dextrorotatory polysaccharide (1%) as the sole source of carbon was sterilized in two portions of 10 ml. in conical flasks (50 ml.) by intermittent steaming at 100° on 3 successive days. The medium was inoculated with a weak spore suspension of *P. luteum* F 6, precautions being taken to avoid removal of nutrients from the agar slope in transferring the spores. The cultures were incubated at 25° (Control cultures with medium containing salts but no added carbohydrate, gave only traces of mycelial growth after 37 days). After incubation for 37 days the polysaccharide medium contained only a trace of imponderable, semi submerged mycelium.

Laevorotatory polysaccharide. A medium corresponding to that described above was prepared, it contained the laevo rotatory polysaccharide (0.5%) as sole source of carbon. Nine portions of the medium (10 ml. each) were inoculated. After incubation for 5 days there was slight superficial growth with green conical areas, and after 37 days the medium was covered with a continuous felt of colourless slimy mycelium, which was harvested, dried and weighed. The yield of mycelium (0.0994 g., i.e. 1.105 g./l. medium) was of the same order as that obtained from media containing lactose (5%), glycerol (5%), or ethanol (2.5%), Table 8. The combined culture filtrate slightly reduced Fehling solution. It had no detectable optical rotation.

Influence of Penicillium luteum polysaccharides on aggregation of soil particles

Geoghegan & Brian (1948a, b) showed that certain bacterial and mould polysaccharides had an aggregating effect on soil particles. The crude polysaccharide from *P. luteum* F 6 described in Table 2, had a definite aggregating effect, although less than that of levans and dextrans (Geoghegan & Brian, 1948b). The crude polysaccharides from strains

nos F 39, 46, 51, 52 and 231 and the pure dextro and laevo rotatory polysaccharides of strain F 6 were tested for soil aggregating properties (Table 9). The products from strains F 46 and 51 had slight aggregating effects, although small compared with the crude product from strain F 6 or levan. The pure dextro and laevo rotatory polysaccharides had much lower activities than the crude product from which they were obtained by alcoholic fractionation.

Table 9 *Influence of Penicillium luteum polysaccharides on aggregation of soil particles*

Polysaccharide	Percentage aggregation when 0.25% of the polysaccharide was applied to soil
Crude products	
Strain F 6*	50.1
Strain F 39	1.8
Strain F 46	4.3
Strain F 51	14.4
Strain F 52	1.3
Strain F 231	1.6
Dextrorotatory polysaccharide, F 6	5.3
Laevorotatory polysaccharide, F 6	23.6
Levan†	90.9
Control	1.3

* Geoghegan & Brian (1948b)

† Geoghegan & Brian (1948a)

DISCUSSION

It is clear from the properties of the dextro and laevo rotatory polysaccharides that they are different from the *P. luteum* products hitherto described. The most important properties are summarized in Table 10 for comparison with those of luteic acid, luteose and galuteose. Anderson & Raistrick (1936) reported the formation in the metabolic products of *P. luteum* Zukal of laevo rotatory polysaccharides, built up of mannose, galactose or fructose units in proportions varying with the age of the culture. Mannose was isolated as the phenylhydrazone (yield, 5%) from some of the crude fractions from our strain F 6 and fructose was detected by partition chromatography, but no pure polysaccharides containing these sugars were isolated. It is concluded that glucose and galactose are the predominant units for polysaccharide synthesis by *P. luteum*. The fungus synthesized a glucose containing polysaccharide from glucose, fructose, mannose, xylose, arabinose and glycerol as sole sources of carbon (Birkinshaw & Raistrick, 1933) and a galactose containing polysaccharide from glucose, galactose, maltose, starch, glycerol and ethanol, but not from acetate, citrate, tartrate, sucrose, dulcitol, mannitol or sorbitol as sole sources of carbon.

The chemical evidence described above supports the conclusion that the polysaccharides are chemical entities. The ultracentrifugal data presented in the

Table 10 *Properties of Penicillium luteum polysaccharides*

Property	Luteic acid (Raistrick & Rintoul, 1931)	Luteose (Raistrick & Rintoul, 1931)	Galuteose (Anderson <i>et al</i> 1939)	Dextrorotatory	Laevorotatory
Specific rotation, $[\alpha]_{481}^{\circ}$	-47 (as Na salt)	-46.4	—	—	—
Specific rotation, $[\alpha]_D^{\circ}$	—	—	-25	+29	-16
Acid equivalent	434.7	Neutral	—	Neutral	800
Component units (100 g)					
D-Glucose (g)	83	100	—	0	64
D-Galactose (g)	—	—	66	88	0
D-Mannose (g)	—	—	20	0	0
Malonic acid (g)	23.4	—	—	—	—
Viscosity of aqueous solution	Viscous	Viscous	Non viscous	Non viscous	Non viscous

following paper (Ogston, 1949) shows, however, that they are polydisperse. The dextrorotatory polysaccharide consists of components of molecular weight of approximately 50,000 to 200,000 and the laevorotatory polysaccharide of components with a mean molecular weight of 15,000. The behaviour of the organism in producing a series of polysaccharides indistinguishable in their chemical properties, but of different molecular complexity, may be related to its power of producing a range of polysaccharide products according to the period of incubation as reported by Anderson & Raistrick (1936).

Stacey (1947) recorded that the biological function of the extracellular mould polysaccharide is unknown, it being generally assumed to act as a reserve carbohydrate. This may be the case with the laevorotatory polysaccharide which sustained about the same amount of mycelial growth as media containing lactose, glycerol or ethanol as sole sources of carbon. The dextrorotatory polysaccharide was not, however, attacked to any significant extent.

P. luteum is common in soils. The marked effect of the crude polysaccharide from strain F 6 on the aggregation of soil particles is of interest. The fungus may take part in producing crumb structure in soil (Geoghegan, 1947). The polysaccharides are hygroscopic and the difficulty of desiccation of their aqueous solutions has been noted, the latter may be

of some biological significance to the fungus in delaying desiccation in soil.

SUMMARY

1 Isolation of two new polysaccharides from strains of *Penicillium luteum* series, grown on Czapek Dox glucose medium, by ethanolic fractionation of dialysed culture filtrates is described.

2 The dextrorotatory neutral polysaccharide, $[\alpha]_D^{30} + 29^{\circ}$ in water (c, 1), was non viscous in aqueous solution. D-Galactose (88%) was the main component sugar, glucose, mannose and fructose were absent. The acetyl and benzoyl derivatives had specific rotations of $[\alpha]_D^{17} + 41^{\circ}$ in chloroform (c, 0.4) and $[\alpha]_D^{18} + 18^{\circ}$ in chloroform (c, 0.6), respectively.

3 The laevorotatory polysaccharide, $[\alpha]_D^{18} - 16^{\circ}$ in water (c, 1), was acidic (acid equivalent, 800). D-Glucose (64%) was the main component sugar, galactose, mannose and fructose were absent. The acetyl polysaccharide was insoluble in the common organic solvents. The benzoyl derivative had a specific rotation of $[\alpha]_D^{16} + 3.3^{\circ}$ in chloroform (c, 0.6).

4 Galactose-containing polysaccharide was synthesized by the mould from glucose, galactose, maltose, starch, glycerol, and ethanol but not from acetate, citrate, tartrate, sucrose, dulcitol, mannitol or sorbitol as sole sources of carbon.

Our thanks are due to Dr M. J. Geoghegan for carrying out the soil aggregation experiments.

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The Sedimentation and Diffusion of Polysaccharides from *Penicillium luteum*, Interpretation of the Results Obtained from Polydisperse Material in the Gouy Diffusiometer

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(Received 18 February 1949)

Samples of the dextro and laevo rotatory polysaccharides, prepared from *Penicillium luteum* by Freeman & Macpherson (1949), were submitted for the examination of their ultracentrifugal sedimentation and diffusion

EXPERIMENTAL

The solid materials were dissolved in, and thoroughly dialysed against, buffer of composition NaCl, 0.2M, KH_2PO_4 , 0.027M, NaHPO₄, 0.027M. The final concentrations were about 1 g/100 ml and were estimated by refractometry against the buffer

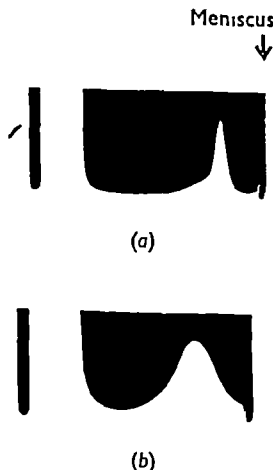


Fig 1 Sedimentation diagrams (a) dextrorotatory polysaccharide 37 min after reaching full speed, (b) laevo rotatory polysaccharide 80 min after reaching full speed (60,000 rev/min.)

Sedimentation measurements Sedimentation was observed in a Svedberg oil turbine ultracentrifuge by the method of Philpot (1938), using the standard conditions of running recommended by Cecil & Ogston (1948)

Both samples proved to be polydisperse. The dextro rotatory material (Fig 1a) was composed of two main fractions: a more slowly sedimenting fraction which appeared to be homogeneous and a more rapidly sedimenting fraction which was heterogeneous. The sedimentation constant of the faster material and the relative proportions of the two fractions could not be determined accurately because the boundaries were not clearly resolved. However, separation of the schlieren curve into two more or less symmetrical parts (Fig 2) gave an approximate estimate of the amounts of the two components. The combined boundary represented only 0.86 of the total refracting material.

The laevo rotatory material (Fig 1b) gave a single boundary, the thickness of which showed that the polysaccharide was polydisperse; it consisted of material sedimenting over a range of rates, symmetrically distributed about a mean value. Integration of this boundary showed that it repre-

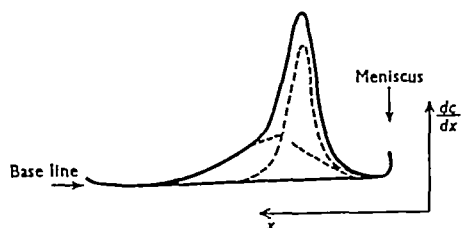


Fig 2 Tracing from sedimentation diagram (full line) of dextrorotatory polysaccharide, 47 min after reaching full speed (60,000 rev/min.), to show analysis into components (broken lines), gradient of concentration dc/dx against position in cell, x

sented only 0.64 of the total refracting material, however, this value may be too low, because the lower limit of the boundary appeared to be reaching the bottom of the cell before its upper limit had fully left the meniscus.

The results are given in Table 1.

Diffusion measurements Diffusion was measured by means of the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948). Ogston (1949) has shown that this method can be used to determine the diffusion constants of a mixture of two

Table 1 *Data obtained from ultracentrifugal sedimentation*

Material	Component	Fraction of total refracting material in ultracentrifuge diagram	Proportion of component in diagram	$\bar{S}_{20(\text{corr})} \times 10^{12}$
Dextrorotatory	(1) Monodisperse	0.86	0.43	4.13
	(2) Polydisperse		0.57	5.2 (approx)
Laevorotatory	Polydisperse	0.64	—	1.79

Table 2 *Data obtained from diffusion*

Material	Component	Proportion of component	$D_{20(\text{corr})} \times 10^7$	Approx mol wt
Dextrorotatory	(1) Monodisperse	0.27	5.5	50,000
	(2) Polydisperse	0.73	1.9	170,000
Laevorotatory	Polydisperse	—	Av 8.32	15,000

homogeneous components. Provided that one of the components is present only in small amount, the error introduced by its polydispersity into the estimate of the diffusion constant of a homogeneous main component is small, however, if, as in the case of the dextrorotatory polysaccharide, a main component is polydisperse, considerable errors are introduced into the estimates of the diffusion constants of both components and of their proportions. The values quoted in Table 2 are, therefore, only rough and it is not surprising that the proportion of the components estimated from the diffusion data differs from that obtained from the sedimentation diagram (Table 1).

Any attempt to analyse the diffusion data given by a highly polydisperse material, such as the laevorotatory polysaccharide, in terms of two homogeneous components, is of little value and could do no more than indicate the range of polydispersity. It would be more useful to estimate the mean diffusion constant, for comparison with the mean sedimentation constant. However, the mean diffusion constant which is obtained from the movement of the outermost interference band by the Gouy method is not the weighted mean diffusion constant, but is given by

$$\frac{1}{\sqrt{D}} = S \frac{\alpha_i}{\sqrt{D_i}},$$

where α_i is the fraction of material having diffusion constant D_i . Use of this value, together with the mean sedimentation constant, to calculate the molecular weight would yield a rather curious mean value. A method has therefore been found, and is described below, for obtaining the arithmetic mean diffusion constant from the Gouy data. The result of this calculation is given in Table 2.

THEORETICAL

Method of obtaining an arithmetic mean diffusion constant by the Gouy method

Where the diffusion boundary is made up of a range of superimposed Gaussian boundaries, if α_i is the fraction of the total refractive increment due to each component having diffusion constant D_i and if v is the total refractive increment (expressed as numbers of wavelengths of phase difference), then the phase difference r of light passing through the

boundary at distances $+x$ and $-x$ from its centre and the angular deflection θ_r of such light at time t are given by

$$r = \frac{2v}{\sqrt{\pi}} S \{ \alpha_i f(z_i) \},$$

where

$$f(z_i) = \int_0^{z_i} e^{-z^2} dz - z_i e^{-z_i^2} \quad \text{and} \quad z_i = x / \sqrt{(4D_i t)},$$

and

$$\theta_r = \lambda S \left\{ \frac{\alpha_i}{\sqrt{(4\pi D_i t)}} e^{-z_i^2} \right\}$$

It follows that

$$\left(\frac{\partial r}{\partial \theta_r} \right)_t = -2x / \lambda.$$

Now

$$\begin{aligned} \bar{D}t &= S (\alpha_i D_i) t = \frac{1}{2} x^2 \\ &= \int_0^\infty \left(\frac{\lambda}{2} \frac{\partial r}{\partial \theta_r} \right)^2 \theta_r dx \bigg/ \int_0^\infty \theta_r dx \\ &= \frac{\lambda^2}{24v} \int_0^\infty \theta_r d \left\{ \frac{\partial r}{\partial \theta_r} \right\}^2 \end{aligned} \quad (1)$$

This quantity can be approximately computed from the Gouy interference pattern by the following procedure. The values of r for the interference minima, from without inwards are $\frac{1}{2}, 1 + \frac{1}{2}, \dots$, the corresponding displacements of the minima from the optic axis, X_r , are measured at a given time t . In addition, the maximal displacement X_{max} is calculated from X_0/β_0 (Coulson *et al.* 1948, Ogston, 1949), which corresponds to $r=0$. $r, X_r, \delta_r, \delta X_r, \frac{\delta r}{\delta X_r}, \left(\frac{\delta r}{\delta X_r} \right)^2$ and $\delta \left(\frac{\delta r}{\delta X_r} \right)^2$ are then tabulated. Since $\theta = X/F$, where F is the focal distance, equation (1) is approximated by

$$\bar{D}t = \frac{F^2 \lambda^2}{24v} S \left\{ X_r \delta \left(\frac{\delta r}{\delta X_r} \right)^2 \right\}$$

This sum is computed over the whole range of interference bands, including the optic axis where $X=0$ and $r=v$, but omitting the interference minimum next to the optic axis, since its proper value of r is uncertain (Kegeles & Gosting, 1947). By thus computing $\bar{D}t$ at two or more values of t , the value of \bar{D} is obtained.

This method was tested on a record obtained with a nearly homogeneous sample of lactoglobulin. The mean diffusion

constant (in buffer at 20°) obtained from $\frac{1}{\sqrt{D}} = S \frac{\alpha}{\sqrt{D}}$ was 7.15×10^{-7} , while the application of the above method gave $\bar{D} = 6.94$ and 7.02×10^{-7} , from two intervals of time. The mean diffusion constant of a mixed solution of lactoglobulin and sucrose (Ogston, 1949) was found to be 10.82×10^{-7} , the expected value being 10.85×10^{-7} .

DISCUSSION

It is clear from the sedimentation diagrams that both polysaccharides are polydisperse, the dextrorotatory consisting of homogeneous and heterogeneous fractions and the laevorotatory being entirely heterogeneous. In addition, in neither case does all the refracting material appear in the sedimentation boundaries, which indicates that a proportion of the material may sediment too quickly or too slowly or may be too highly polydisperse to contribute to the boundary diagrams. Thus, while the mean sedimentation constants of the material can be regarded as established, in view of the uncertainties and errors discussed above neither the proportions of the components nor their diffusion constants should be regarded as more than rough estimates, and the same is true of the estimates of molecular weights,

given in Table 2. These were derived from the sedimentation and diffusion constants assuming a value of 0.62 for the partial specific volumes.

The value of the mean diffusion constant of the laevorotatory polysaccharide, calculated by the method described, would give a reliable estimate of its mean molecular weight if it were certain that the average applied to the same range of material as does the estimate of the sedimentation constant. The fact that only 0.64 of the refracting material appears in the sedimentation boundary shows that this may not be so.

SUMMARY

1. Measurements of the sedimentation and diffusion of two samples of polysaccharide from *Penicillium luteum* are described.

2. A new method is given for analysing the diffusion data obtained with the Gouy diffusometer, so as to obtain a value of the arithmetic mean diffusion constant which is comparable with the mean sedimentation constant.

3. Approximate values for the amounts and constants of the components of the polysaccharides have been deduced.

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Metabolic Products of *Trichothecium roseum* Link

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(Received 22 February 1949)

The work described in this paper was carried out independently at Stevenston and the London School of Hygiene and Tropical Medicine. When the separate investigations were communicated to the Biochemical Society (Freeman & Morrison, 1948; Michael, 1948) the results from the two laboratories were found to be so similar that it was decided to present a joint account of the work. This paper deals with the isolation of three crystalline metabolic products of *Trichothecium roseum* which had not hitherto been described. Two of these products which are present mainly in the mycelium, and only in smaller amounts in the culture fluid, were named

rosein I and rosein II, while the name rosein III is proposed for the third compound which has been found only in the culture fluid. The three new products are additional to trichothecin, the antifungal substance which was described by Freeman & Morrison (1949a).

It has been known for many years that fruits attacked by the pink rot caused by *T. roseum* Link contain a bitter principle. The latter was isolated in the form of a crude syrup and its solubility described by Iwanoff (1904). Antagonism between *T. roseum* and certain plant pathogenic fungi was reported by Whetzel (1909), Boning (1933), Koch (1934) and

Greaney & Machacek (1935) Koch (1934) stated that *T. roseum* actively parasitized the stroma of *Dibotryon morbosum* in the black knot disease of various hosts. Greaney & Machacek (1935) found that the fungus was antagonistic to *Helminthosporium sativum* when the two organisms were grown together in soil in the presence of cereal seedlings. The presence of an antifungal compound in culture filtrates of *Trichothecium roseum* was reported by Brian & Hemming (1947). The substance, trichothecin, responsible for the antifungal activity was isolated, and some of its chemical properties described by Freeman & Morrison (1949*a*). Its biological properties were described by Freeman & Morrison (1949*b*). Rosein I and crude rosein III were encountered as by-products in the isolation of trichothecin by Freeman & Morrison (1949*a*) and referred to in that paper as 'crystalline precipitate (II)' and 'precipitate (I)' respectively.

The mould was incubated for 3–4 weeks on Czapek-Dox medium which was enriched with corn-steep liquor. Extraction of the culture fluid with chloroform yielded a syrupy mixture from which rosein III was obtained as a colourless crystalline product by repeated precipitation with light petroleum from chloroform, followed by fractionation on an alumina column in chloroform-carbon tetrachloride. The petroleum mother liquors were evaporated and fractionated in ether by passing through an alumina column. The first fractions, on recrystallization from light petroleum containing some chloroform, yielded small amounts of roseins I and II, in the mother liquors of these products the bulk of the trichothecin was found.

Extraction of the dried and powdered mycelium with solvents yielded a mixture of two crystalline products, roseins I and II, the former in approximately double the amount of the latter. These products were accompanied in the extracts by considerable amounts of lipids.

In the London School of Hygiene and Tropical Medicine method, the extraction was carried out in a Soxhlet extractor with light petroleum. On concentrating and cooling the extract, a mixture of roseins I and II crystallized in yields of 2–3 % of the weight of mycelium. Separation of the two substances was effected by treatment with cold ethanol which leaves rosein I, m.p. 210°, undissolved, while rosein II, m.p. 186°, crystallizes from the mother liquors on addition of water.

In the Nobel Division method, boiling chloroform was used as solvent. Treatment of the concentrated extract with light petroleum afforded rosein I. On evaporation of the mother liquors and treatment with aqueous ethanol, rosein II was obtained.

Rosein I ($C_{19}H_{26}O_3$, m.p. 210°), which is slightly soluble in cold ethanol, crystallizes from this solvent in colourless tetrahedra. It is optically active, its

specific rotation in chloroform being $[\alpha]_D -112.5^\circ$. It is a neutral compound of ketonic character, as shown by the formation of a 2,4-dinitrophenyl hydrazone and an oxime. Under the influence of hot alkali, it is rearranged to a lactone, *isorosein* I ($C_{19}H_{26}O_3$, m.p. 144°), which is no longer ketonic, but which possesses a hydroxyl group which can be benzoylated. Potassium permanganate at room temperature oxidizes rosein I to a monobasic acid, $C_{16}H_{21}O_4$, m.p. 242°.

Rosein II ($C_{19}H_{26}O_3$, m.p. 186°) crystallizes from aqueous ethanol or from toluene in colourless fibrous needles. It possesses weak optical activity, its specific rotation $[\alpha]_D$ in chloroform being $+5.9^\circ$. It has no ketonic or hydroxyl groups, but its properties correspond to those of a lactone. It is soluble in hot alkali and is recovered unchanged on acidification. Oxidation with potassium permanganate in acetone at room temperature gave a monobasic acid $C_{16}H_{23}O_5$. Rosein II was obtained from cultures on the enriched Czapek-Dox medium, but was not detected in cultures on Raulin-Thom medium.

Rosein III ($C_{20}H_{28}O_4$, m.p. 221°) crystallizes from toluene in colourless plates. It has a ketonic character as shown by the formation of a 2,4-dinitrophenylhydrazone. Hot alkali converts rosein III into an isomeric lactone, *isorosein* III, $C_{20}H_{28}O_4$, m.p. 155°.

During the isolation of one of the strains used in this work (BB 105*a*) it was observed that an apple infected with the mould developed an intensely bitter taste, which permeated the whole of the fruit even when only a small area was infected. Filtrates from cultures of the mould on liquid media also possessed a bitter taste. An amorphous solid, similar in properties to Iwanoff's (1904) bitter principle, was isolated by ether extraction of the culture filtrate; trichothecin was shown to be one of its principal bitter components.

Antibiotic properties of the metabolic products
While roseins I and III were devoid of antibiotic activity against the test organisms, rosein II inhibited the growth of *Bacillus subtilis* at a concentration of 5 mg/l. It inhibited *Mycobacterium phlei* at 1:81,000, was only slightly active against *M. tuberculosis* and had no effect on *M. butyricum*.

EXPERIMENTAL

Organisms and cultural conditions

The organisms used for most of the work in Nobel Division were strains of *Trichothecium roseum* Link isolated from mouldy dead wood, laboratory catalogue numbers F 109 and F 227. They are described in detail by Freeman & Morrison (1949*b*). The strains used at the London School of Hygiene and Tropical Medicine were L S H T M, catalogue number BB 105*a*, isolated from an apple and identified by Mr G. Smith in 1945 and L S H T M, catalogue number 95, isolated from a tomato by Mrs. Marcus in 1947.

The strains all grew well on Czapek Dox agar and beer wort agar and rapidly developed the typical pink conidial areas. Preliminary experiments showed that on liquid Czapek Dox or Raulin Thom media growth and sporulation were slow and sparse. Czapek Dox solution (350 ml) in 1 l. conical flasks was inoculated with a heavy spore suspension and incubated at 24°. After 14 days, a thin mycelium had formed and pink conidial areas began to appear, at this stage the culture filtrate still contained approximately 4% glucose and its pH was 6.4. After incubation for 31 days the mycelium was thin, but well covered with spores. The pale yellow culture filtrate contained 3.2% glucose and its pH was 7.3.

After incubation for 7 days cultures on Raulin Thom solution developed a white mycelium which had a tendency to sink below the surface of the liquid. Weak and patchy sporulation appeared after 24 days.

Czapek Dox medium, which was enriched by the addition of 1% corn steep liquor (50% total solids), sustained more rapid and abundant growth and spore formation. Czapek Dox solution (350 ml) + corn steep liquor (3.5 ml) in 1 l. conical flasks was inoculated with a heavy spore suspension and incubated at 24°. After 6 days a thick mycelium had formed and spore formation was beginning. After 14 days the mycelium was completely covered by pink conidial areas. The cultures were harvested after incubation for 21 days, when the pale yellow culture filtrate contained 0.7% glucose and its pH was 7.6.

The culture medium used in Nobel Division was based on the Czapek Dox formula in which NaNO_3 was replaced by the equivalent quantity of ammonium tartrate and with the addition of 1% corn steep liquor. The medium used at L.S.H.T.M. was Czapek Dox solution with the addition of 1% corn steep liquor.

Isolation of roseins I and II

Nobel Division method

Rosein I The medium used contained ammonium tartrate (2 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g), K_2HPO_4 (1.0 g), KCl

(0.5 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g), crude commercial glucose (50 g), corn steep liquor (10 ml) in water to 1 l. It was based on Czapek's formula (as modified by Dox, 1910), in which ammonium tartrate was substituted for NaNO_3 , and corn steep liquor was added as a supplementary nutrient. Crude commercial glucose ('glucose chips') was found to be a satisfactory source of sugar.

The medium (300 ml) in 'Glaxo' bottles was sterilized by autoclaving. The reaction was adjusted to pH 5.0. The bottles were inoculated with a heavy spore suspension and incubated at 25° for 28 days in darkness.

The mycelium (strain F 109) was harvested and separated from the culture fluid by filtration through fluted filter paper, washed with cold water and dried in a current of air at 20°. Air dried, powdered mycelium (159.2 g from 15 l. of medium) was extracted three times under reflux for 0.5 hr with boiling CHCl_3 (500 ml). The combined extracts were concentrated, yielding an orange yellow oily residue (36.3 g, 22.8% of mycelium). The residue was treated with light petroleum (b.p. 60–80°, 250 ml) and a crystalline precipitate of crude rosein I (6.58 g, 4.13% of mycelium) slowly formed. The precipitate was collected, washed with light petroleum and extracted with cold ether. The mother liquor (A) and the ether extract (B) were retained. The crude rosein I was dissolved in CHCl_3 (5 ml) and the solution passed through a column (20 × 1.5 cm) of activated alumina in CHCl_3 . The solvent was evaporated from the eluate and the residue (5.18 g) was dissolved in the minimum volume of CHCl_3 (5 ml) and diluted with ether (30 ml). Slightly impure rosein I (m.p. 208°, 4.53 g) crystallized out. The pure substance was obtained after one recrystallization from ethanol (4.11 g, 2.58% of mycelium, m.p. 210°) in the form of colourless tetrahedra.

Rosein II The mother liquor (A), consisting mainly of a solution of lipids in light petroleum, was evaporated to dryness and the orange coloured residue (29.71 g) extracted for a few minutes with boiling 75% (v/v) aqueous ethanol (100 ml). The extract was cooled, shaken with light petroleum (b.p. 60–80°, 30 ml) and the ethanol layer separated and combined with the ether extract (B). On evaporation of

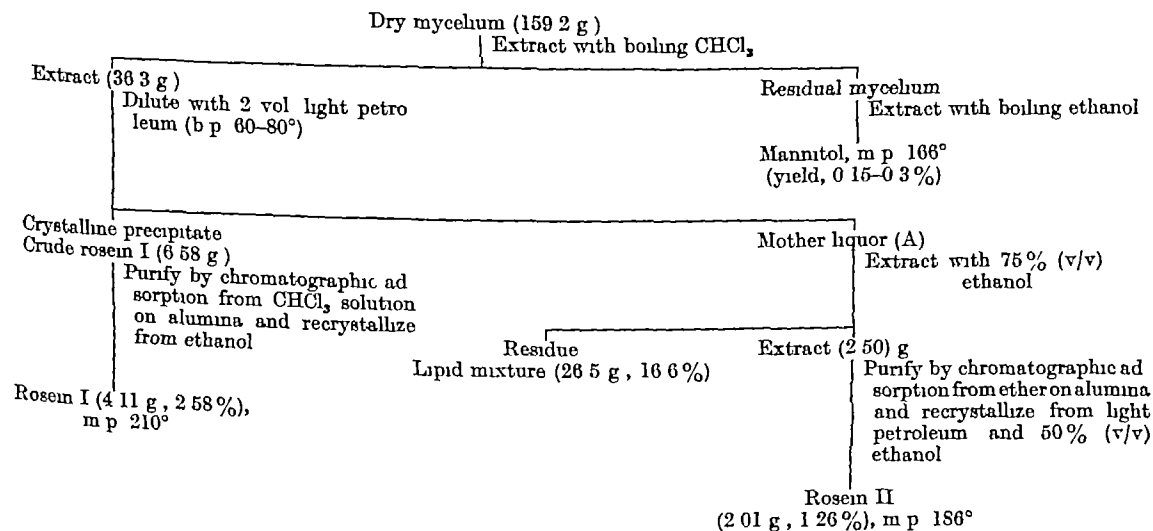


Fig 1 Isolation of roseins I and II from mycelium

the combined ethanol and ether solutions nearly to dryness crude rosein II crystallized (2.50 g). It was dissolved in CHCl_3 , boiled with a little activated charcoal and filtered. The residue, formed on evaporation of the filtrate, was recrystallized first from light petroleum (b.p. 60–80°) and then from 50% (v/v) aqueous ethanol giving pure rosein II (2.01 g, 1.26% of mycelium, m.p. 186°) in the form of colourless fibrous needles. Passage of an ether solution through a column of activated alumina was used in purification of some batches of crude rosein II.

The residue remaining after isolation of roseins I and II consisted mainly of a mixture of lipids, partially crystalline at 20°.

The fractionation of the mycelial products is summarized in Fig. 1.

London School of Hygiene and Tropical Medicine method

In several experiments 1 l. conical flasks (50), each containing Czapek Dox solution (350 ml) and corn steep liquor (3.5 ml) were inoculated and incubated for 21–26 days at 24°. At the end of this period the metabolism solution was separated from the mycelium by filtration and the latter well pressed, washed with water, dried *in vacuo* at 35° and finally ground to a fine powder. The yield from 50 flasks was 200–210 g dry mycelium.

The culture filtrate was a clear, pale yellow liquid, pH 7.6. It contained 0.5–0.7% residual glucose. It gave no reaction with FeCl_3 . The solution had an intensely bitter taste. After cooling to 5° for 24 hr, small amounts (0.1–0.2 g from 1 l.) of colourless crystals had separated. They were filtered off, washed with water and dried *in vacuo*. The crystalline product consisted of a mixture of needles and tetrahedra identical with specimens of roseins I and II isolated from the mycelium.

The culture filtrate was three times extracted with $\frac{1}{3}$ vol. of ether. The extract was filtered through a dry filter, evaporated to small volume, and then allowed to evaporate slowly. Small amounts (0.1–0.2 g) of the same mixture of crystals separated. They were filtered off, washed with a little ether and dried *in vacuo*. The filtrate on evaporation to dryness gave an amorphous, buff coloured powder (2 g), having an intensely bitter taste. The product was slightly soluble in water, soluble in dilute NaOH giving a frothy solution and soluble in ethanol, acetone and ether.

Isolation and separation of metabolic products from mycelium. The dried, powdered mycelium was extracted in a Soxhlet extractor with light petroleum (b.p. 40–60°). After 3–4 hr colourless crystals began to appear in the receiver. After 7 hr the extract was yellow and had a strong smell of a fatty or oily character. At this stage the receiver was changed and extraction continued for four to five periods of 7 hr. The extracts were separately concentrated to small volume, kept at 0° for 16 hr and the crystalline deposit filtered off, washed well with cold light petroleum and dried *in vacuo*. The product consisted of varying mixtures of colourless needles and tetrahedra. On evaporation of the combined mother liquors to small volume a further crop of crystals was obtained.

A typical extraction of dry mycelium (42 g) gave from successive 7 hr extractions 0.8 g crystals, mainly needles, 0.3 g crystals, a mixture, 0.1 g crystals, mainly tetrahedra, and 0.05 g crystals, mainly tetrahedra (total 1.25 g, 2.98% of mycelium). The average yield, throughout the work, of the mixed crystalline product was 2–3% of the dry mycelium,

equivalent to 0.3–0.4 g/l of culture medium. The final mother liquors of the crystalline products, when freed from solvent, were semi solid yellow oils.

The mixed crystalline product was separated into its components by extraction with cold ethanol in which rosein II was readily soluble, whilst rosein I was only slightly soluble. The mixture (2.85 g) was suspended in ethanol (30 ml) and stirred for 2 min at room temperature. The colourless extract was filtered off and the insoluble residue washed with two 5 ml portions of ethanol. The insoluble fraction was dried *in vacuo*; it consisted of rosein I, colourless tetrahedra (1.67 g, m.p. 210°).

The ethanolic mother liquor and washings of rosein I were combined, warmed to 60° and diluted with an equal volume of water at 60°. On cooling, colourless needles of rosein II separated. After standing at 0° for 16 hr, the product was filtered off, washed with ice cold 50% (v/v) aqueous ethanol and dried *in vacuo*. Yield of rosein II, 0.8 g, m.p. 183°. The melting point of the product was raised to 186° on recrystallization from toluene.

The mother liquor of rosein II was freed from ethanol by evaporation and cooled to 0° for 16 hr. A deposit (0.3 g) of a mixture of roseins I and II separated and was worked up with a subsequent batch. The initial crystalline mixture contained approximately two parts of rosein I and one part of rosein II.

Isolation of rosein III from culture filtrate

Rosein III was not obtained from the mycelium. It was extracted, together with trichothecm and small amounts of roseins I and II, by CHCl_3 from the culture filtrate (strain 227) and isolated by the following procedure.

The culture filtrate in lots of 1 l. was twice extracted with CHCl_3 (200 ml). The combined extracts were evaporated to dryness under reduced pressure and the dried extract from 80 l. of culture filtrate was obtained as a brown gummy solid (21.23 g). The latter was dissolved in CHCl_3 (20 ml) and the solution diluted with ether (450 ml). The resultant precipitate was rejected and the supernatant solution evaporated to dryness. The residue was redissolved in CHCl_3 (20 ml) and the solution diluted with light petroleum (b.p. 60–80°, 450 ml). The gummy precipitate which formed was separated by decantation, redissolved in CHCl_3 (10 ml.) and reprecipitated by addition of light petroleum as above. The extracts were combined and on evaporation to dryness gave 11.65 g of an amorphous residue (A). The precipitate (B, 7.68 g) was dissolved in 50% (v/v) $\text{CCl}_4/\text{CHCl}_3$ mixture and fractionated chromatographically on an alumina column (30 × 3.8 cm) which was developed with a mixture of CCl_4 and CHCl_3 . The eluate was collected in 50 ml fractions, which after evaporation of the solvent were treated with a little ether. The crystalline fractions were combined (4.72 g) and dissolved in a little CHCl_3 . On addition of ether, rosein III crystallized out. The pure compound (3.68 g, m.p. 221°) was obtained after recrystallization from toluene as colourless plates.

The petroleum ether soluble portion (A, 11.65 g) containing trichothecm was dissolved in ether and fractionated chromatographically on an activated alumina column. The eluate was collected in 100 ml fractions, the first three of which contained the bulk of the trichothecm. After evaporation of the solvent (ether) the fractions were dissolved in light petroleum (b.p. 60–80°) containing 5% (v/v) of CHCl_3 . After standing at 5° for 16 hr, fraction II gave a crystalline deposit of rosein I (0.25 g) and fraction III gave rosein II

(0.11 g) After separation of these crystalline precipitates, the combined mother liquors were used for preparation of trichothecm. Fractions IV, V, VI and VII gave crystalline deposits of crude rosem III, totalling 2.11 g. The pure product (m.p. 221°) was obtained on recrystallization.

It was found to be most convenient and efficient to separate the fractions of (A) which were free from trichothecm (as determined by antifungal tests) and combine them with (B), for chromatographic purification of rosem III, as shown in Fig. 2. The total yield of rosem III was about 60 mg/l. of culture filtrate.

in CHCl_3 (c, 1). Nitrogen, halogens and sulphur were absent (Found (Weiler and Strauss) C, 75.4, 75.4, H, 8.9, 8.8, mol. wt. (cryoscopic in benzene), 302, (Rast), 316 methoxyl, nil, active hydrogen, nil. $\text{C}_{19}\text{H}_{28}\text{O}_2$ requires C, 75.5, H, 8.6%, mol. wt. 302).

Rosem I dissolved in conc. H_2SO_4 to give a pale orange yellow solution. The compound gave no colour reactions for sterols. A solution in aqueous ethanol was neutral, gave no reaction with FeCl_3 and did not reduce Fehling solution or Tollens ammoniacal AgNO_3 reagent. There was no reaction with Br_2 in CCl_4 . An acetone solution of KMnO_4 was slowly

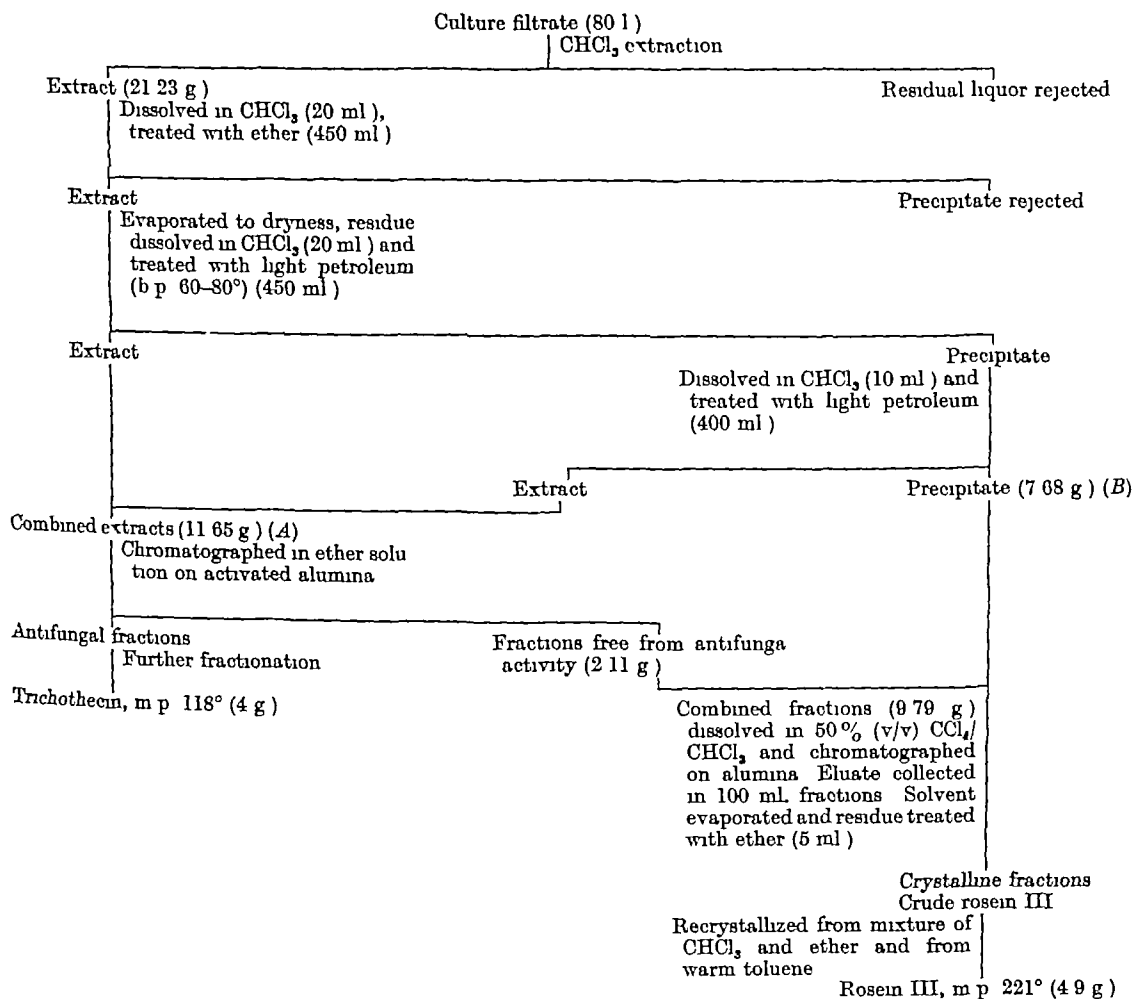


Fig. 2 Isolation of rosem III

Properties and reactions of metabolic products

Rosem I

Rosem I crystallized from hot ethanol in colourless tetrahedra, m.p. 210°. It was readily soluble in CHCl_3 or acetone and was fairly soluble in benzene, slightly soluble in ether or ethanol, very slightly soluble in cold light petroleum and practically insoluble in water, cold dilute NaOH and dilute HCl . It was optically active $[\alpha]_D^{24} - 112.5^\circ$, $[\alpha]_{5461}^{24} - 140^\circ$

decolorized at room temperature. Alkaline KMnO_4 was not decolorized at room temperature, but was rapidly decolorized on warming. Rosem I gave no benzoyl derivative on treatment with benzoyl chloride and pyridine.

Rosem I 2,4-dinitrophenylhydrazone Rosem I (100 mg) was dissolved in glacial acetic acid (5 ml) and mixed with 2,4-dinitrophenylhydrazine (150 mg) in glacial acetic acid (10 ml). After 18 hr, the mixture was diluted with 2N HCl and the crystalline precipitate filtered off and washed first with 2N HCl and then with water until neutral. The crude

product (147 mg) was dissolved in CCl_4 and purified chromatographically on activated alumina. The main band and several minor bands were developed and eluted by gradually increasing the proportion of CHCl_3 in the solvent. The main fraction (120 mg) crystallized from aqueous ethanol in the form of yellow needles, m p 239° .

The same product was obtained by treatment of rosein I (100 mg) in absolute ethanol with 1.5 ml Brady reagent (1 g 2,4-dinitrophenylhydrazine in 2 ml conc H_2SO_4 and 15 ml ethanol). Yellow needles began to crystallize after 30 min, and the product was collected after 20 hr at room temperature. The hydrazone in ethanolic solution gave an intense red colour with strong alkali. (Found C, 62.7, 62.3, H, 6.3, 6.3, N 11.3, 12.5. $\text{C}_{25}\text{H}_{30}\text{O}_8\text{N}_4$ requires C, 62.2, H, 6.2, N, 11.6%.)

Rosein I oxime Rosein I (100 mg) dissolved in warm ethanol (5 ml.) was treated with hydroxylamine hydrochloride (30 mg) and anhydrous sodium acetate (200 mg). The mixture was kept at 60° for 1 hr and most of the ethanol distilled off. The residue was diluted with water and extracted with ether. The extract was washed with water, dried over Na_2SO_4 and evaporated to dryness. The residue (96 mg) was recrystallized from aqueous ethanol and formed colourless prisms, m p 215° (Found C, 71.7, H, 8.5, N, 4.3, 4.5. $\text{C}_{19}\text{H}_{27}\text{O}_5\text{N}$ requires C, 71.9, H, 8.5, N, 4.4%.)

Isorosein I Rosein I (220.8 mg) was heated under reflux for 6 hr with 0.1 N ethanolic KOH (20 ml). The solution was cooled, diluted with water and titrated with 0.1 N HCl (Found 220.8 mg rosein I equiv to 7.56 ml 0.1 N HCl, saponification value, 292 (theory, 302)). The bulk of the ethanol was distilled off and the residue made acid to congo red indicator by addition of conc HCl. The resultant colourless precipitate was extracted with ether. The extract crystallized slowly on evaporation of the solvent giving colourless crystalline isorosein I (190 mg, 86.1% yield). The product was recrystallized from light petroleum (b p $60\text{--}80^\circ$) and then from 50% (v/v) aqueous ethanol in the form of colourless prisms, m p 144° . Isorosein I was readily soluble in cold ethanol, ether, CHCl_3 , acetone and benzene, slightly soluble in light petroleum and insoluble in water. It dissolved slowly in hot 2 N NaOH. No precipitate appeared on cooling. On addition to the cold alkaline solution of an equal volume of phosphate buffer, pH 6, no precipitate appeared. On acidifying with 2 N HCl, isorosein I, m p 143° , crystallized. It did not dissolve in NaHCO_3 solution and had no acidic properties. (Found C, 75.7, 75.4, H, 8.5, 8.9, mol wt (cryoscopic in benzene), 280. $\text{C}_{16}\text{H}_{22}\text{O}_3$ requires C, 75.5, H, 8.6%, mol wt 302.) $[\alpha]_{\text{D}}^{20} + 40^\circ$ (c, 1 in ethanol). Isorosein I did not react with hydroxylamine or with 2,4-dinitrophenylhydrazine.

Monobenzoyl isorosein I Isorosein I (200 mg), dissolved in pyridine (5 ml.), was treated with benzoyl chloride (500 mg). After standing at 20° for several days, the mixture was poured into ice cold dilute HCl and extracted with ether. The extract was washed first with water, then with dilute NaOH and finally with water until neutral. On evaporation of the solvent, the residue crystallized slowly. It was decolorized by solution in ether and passage through a column of activated alumina. Recrystallization from aqueous ethanol gave colourless needles of monobenzoyl isorosein I, m p 166° (180 mg) (Found C, 75.7, H, 7.6. $\text{C}_{26}\text{H}_{30}\text{O}_4$ requires C, 76.9, H, 7.4%.)

Hydrolysis of monobenzoyl isorosein I Monobenzoyl isorosein I (81 mg) was dissolved in ethanol (10 ml) and

heated under reflux with 0.1 N KOH (10 ml). Titration with 0.1 N HCl showed that roughly 2 equiv of alkali had been taken up, which was in accordance with the hypothesis that 1 equiv was taken up in hydrolysis of the benzoate and 1 equiv in hydrolysis of a lactone ring. The solution was made acid to congo red and allowed to stand for 24 hr. A slight excess of NaHCO_3 was then added and the solution extracted with ether. The extract was washed with water, dried over Na_2SO_4 and the solvent evaporated off. The crystalline residue was recrystallized from ethanol giving colourless crystals, m p $213\text{--}214^\circ$, in insufficient quantity for analysis.

Permanganate oxidation of rosein I Rosein I (50 mg) was dissolved in a 1% solution of KMnO_4 in acetone (5 ml.). After 1 hr the purple colour had disappeared and MnO_2 had been precipitated. A further 5 ml of KMnO_4 solution was added, after standing for 16 hr the purple colour had again been discharged. The MnO_2 was filtered off, washed well with acetone and dried. On evaporating the filtrate and washings, rosein I (37 mg) was recovered. The MnO_2 was twice extracted with 10 ml portions of boiling water. On acidification of the colourless extract with HCl, sheaves of colourless needles soon crystallized out. They were collected after 16 hr at 0° , m p 242° (7.5 mg).

In a second experiment, rosein I (50 mg) was treated with 1% KMnO_4 in acetone (25 ml). After 3 days at room temperature, the permanganate was nearly decolorized and the solution was decolorized by passage of SO_2 . The mixture was worked up as described above and the same product isolated (8.5 mg). (Found on product dried to constant weight *in vacuo* at 60° C, 68.2, H, 8.1, acid equiv 5.4 mg required 0.18 ml 0.1 N NaOH (cold or hot) giving equiv for one acidic group, 300. $\text{C}_{16}\text{H}_{24}\text{O}_4$ requires C, 68.6, H, 8.6%, equiv 280.)

Rosein II

Rosein II crystallized from light petroleum (b p $60\text{--}80^\circ$), toluene or 50% (v/v) aqueous ethanol in long fibrous needles, m p 186° . It was practically insoluble in water and cold 2 N NaOH, only slightly soluble in light petroleum, but readily soluble in most other organic solvents, such as ether, acetone, ethanol and CHCl_3 . Its solution in conc H_2SO_4 was deep orange yellow. There was no reaction with ethanolic FeCl_3 . The Salkowski and Liebermann-Burchard reactions for sterols were negative. A solution of rosein II in aqueous ethanol was neutral and had no reducing properties. A 2,4-dinitrophenylhydrazone could not be prepared. Rosein II was weakly dextrorotatory, $[\alpha]_{\text{D}}^{23} + 5.9^\circ$ in CHCl_3 (c, 2), $[\alpha]_{\text{D}}^{20} + 7.5^\circ$ in ethanol (c, 1). Nitrogen, sulphur and halogens were absent. (Found C, 75.2, 75.3, 75.4, H, 9.3, 9.5, 9.3, mol wt (Rast) 278, 337, methoxyl, nil, active hydrogen, nil. $\text{C}_{19}\text{H}_{24}\text{O}_3$ requires C, 75.0, H, 9.2%, mol wt, 304.)

Action of alkali Rosein II (0.15 g) was dissolved in ethanol (50 ml.) and 10 N NaOH (3 ml) added. The clear solution was heated under reflux on a steam bath for 2 hr. After cooling and removing about half the ethanol *in vacuo*, the solution was diluted with water (30 ml) and acidified with 2 N HCl, rosein II (0.135 g, m p 186°) crystallized as colourless needles.

Permanganate oxidation Rosein II (1.008 g) was dissolved in a 1% solution of KMnO_4 in acetone (200 ml). After 16 hr at 25° the purple colour had almost disappeared. The precipitated MnO_2 was filtered off, washed well with

acetone and dried. On evaporating the acetone filtrate and washings unchanged rosein II (0.3157 g, m.p. 186°) was recovered.

The dried MnO_2 was twice extracted with boiling water (200 ml.) On cooling there was a small deposit (7.5 mg) from the first aqueous extract. The filtrate was acidified by addition of conc. HCl (2.5 ml) giving an immediate amorphous colourless precipitate of oxidation product (0.2672 g). The latter was crystallized from 50% (v/v) aqueous ethanol (17 ml) yielding colourless hexagonal plates (0.098 g, m.p. 206°) (Found C, 64.1, 64.2, H, 8.5, 8.7. $C_{16}H_{14}O_8$ requires C, 64.5, H, 8.8%). Acid equiv. 32.6 mg required 2.14 ml 0.05N NaOH (hot or cold) in aqueous ethanolic solution giving equiv. for one acidic group, 305 (theory 298).

Rosein III

Rosein III crystallized from toluene in colourless plates, m.p. 221°. It was only very slightly soluble in water or light petroleum (b.p. 60–80°), slightly soluble in ether and readily soluble in $CHCl_3$ or ethanol. $[\alpha]_D^{19} = -124^\circ$ in $CHCl_3$ (c, 1). Nitrogen, sulphur and halogens were absent (Found C, 72.1, 72.5, 72.5, H, 8.3, 8.5, 8.5, methoxyl, nil, mol wt (Rast) 339. $C_{20}H_{28}O_4$ requires C, 72.3, H, 8.4, mol wt, 332).

In aqueous ethanolic solution, rosein III was neutral, had no reducing properties and gave no reaction with $FeCl_3$. On treatment with acetic anhydride in glacial acetic acid, the compound (m.p. 221°) was recovered unchanged.

Rosein III 2,4-dinitrophenylhydrazone Rosein III (0.110 g) in ethanol (2 ml) was treated with Brady reagent (1.5 ml) as described above. The crude hydrazone (0.167 g) was purified chromatographically from ether solution on activated alumina. The main fraction was crystallized from ethanol as yellow fibrous needles (0.107 g, m.p. 280° decomp.) (Found C, 60.2, H, 6.2, N, 11.3. $C_{28}H_{32}O_7N_4$ requires C, 60.8, H, 6.2, N, 11.0%).

isoRosein III Rosein III (0.5 g) dissolved in 6% methanolic KOH (25 ml) was heated under reflux for 4 hr. The solution was cooled, diluted with water and the methanol distilled off *in vacuo*. The residue was acidified with HCl and extracted with $CHCl_3$. The extract was washed, dried over Na_2SO_4 and evaporated almost to dryness. The residue was a pale yellow syrup which crystallized slowly. The crude product was recrystallized from toluene and obtained as colourless microcrystalline needles (0.135 g, m.p. 155°) (Found C, 71.9, H, 8.3, mol wt (Rast) 272. $C_{20}H_{28}O_4$ requires C, 72.3, H, 8.4%, mol wt, 332).

Biological properties of metabolic products

Roseins I, II and III were tested for antibiotic activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Penicillium digitatum*. The metabolic products in 0.1% solution in ethanol were placed in porcelain cylinders embedded in suitable media in Petri dishes, the media having been seeded with one of the test organisms. The plates were examined for zones of inhibition after 24 hr. incubation at 32° in the case of the bacterial cultures and incubation for 5 days at 25° for *P. digitatum*. There was no inhibition of growth except that of *B. subtilis* by rosein II, which gave a zone of partial inhibition of 2.5 cm diameter. In serial dilution tests *B. subtilis* grew normally in nutrient broth medium containing 2.5 mg rosein II/l, but there was complete inhibition at 5.0 mg/l.

Weak antibacterial activity against *B. subtilis* was observed in filtrates of cultures of *T. roseum* F 109, grown on Czapek Dox medium, by the cylinder plate technique at an early stage in the work. This is now shown to be due to the presence of rosein II. Dilutions of the culture filtrates were only feebly active since the solubility of rosein II in water at 20° does not greatly exceed its limiting antibacterial concentration.

The effect of rosein II on the growth of three species of acid fast bacteria was investigated by Dr A. R. Martin. The test against *Mycobacterium tuberculosis* was carried out by incorporating the substance in an agar medium and inoculating the organism on to the surface. After incubation for 14 days the organism grew poorly in medium containing a concentration of 1/3000 of rosein II and some inhibition was still apparent at a concentration of 1/81,000. *Mycobacterium phlei* and *Mycobacterium butyricum* were grown on liquid media in which rosein II had been dispersed and the observations made after incubation for 2 days. Growth of the former was completely inhibited at 1 in 81,000, but normal growth took place at 1 in 243,000. Normal growth of the latter took place in the presence of 1 part rosein II in 1000.

Isolation of trichothecin as a component of the bitter principle of *Trichothecium roseum*

Iwanoff (1904) first reported that the metabolic products of *T. roseum* contained a very bitter substance. This was also noticed by the present authors and by Yasue (1948, private communication), but the bitter components have not hitherto been characterized.

The culture filtrate from 50 cultures of strain BB 105a (17.5 l) was three times extracted with 2 l lots of ether. On evaporation, an amorphous, buff coloured powder (2 g) of intensely bitter taste was obtained. A sample (36.2 mg) was dissolved in ethanol (1 ml) and diluted to 100 ml with water giving an opalescent solution. After suitable dilution, the solution was assayed for antifungal activity by the *Penicillium digitatum* spore germination technique which showed that a powerful antifungal compound was present. The ether extract (1.48 g) was fractionated by the method described by Freeman & Morrison (1949a) for the isolation of trichothecin. The combined light petroleum (b.p. 60–80°) extracts (0.80 g) were dissolved in ether (10 ml) and fractionated on a column of activated alumina (25 × 2.0 cm). The chromatogram was developed with ether and the eluate collected in 100 ml. fractions. These were evaporated to dryness and treated with light petroleum containing 5% (v/v) $CHCl_3$ (5 ml). Fraction 2 gave crystalline trichothecin (57.5 mg), m.p. and mixed m.p. 118°. Fractions 5 + 6 gave crystalline rosein II (14.2 mg), m.p. 186°.

The fraction insoluble in light petroleum was fractionated as described in the preparation of rosein III, but only traces of uncharacterized crystalline fractions were obtained. No rosein III was detected.

Tasting tests by four independent observers showed that the water insoluble compounds, roseins I and II, were tasteless, rosein III was slightly bitter, and trichothecin and isotrichothecin (hitherto referred to as the alkaline hydrolysis product of trichothecin by Freeman & Morrison, 1949a) were very bitter.

The available evidence suggests that trichothecin is responsible for part, at least, of the bitter taste of *T. roseum* culture filtrates and the bitterness of fruits attacked by the fungus.

*Carbon balance of metabolic products of
Trichothecium roseum*

In order to determine whether trichothecin and roseins I, II and III, isolated in yields of 50, 3, 1 and 61 mg/l respectively from modified Czapek Dox medium, represented the main soluble metabolic products of *T. roseum*, partial carbon balance sheets of four strains grown on Czapek Dox medium were prepared. The results (Table 1) showed that of the carbon in the initial glucose, 7–15% remained in the culture filtrate in the form of non volatile components other than glucose and 11–18% was found in the mycelium. There was no production of acidic substances as shown by a steady rise of pH during incubation, until a final value of pH 7–9 was reached. The observations were based on the bulked filtrates from two 'Glaxo' bottle cultures (350 ml), of each strain, which were diluted to 1 l for analysis, initial pH 6.6. Portions (100 ml) of the diluted filtrates were twice extracted with CHCl_3 (50 ml), the aqueous fraction freed from CHCl_3 by evaporation and carbon determined by the wet combustion method of Houghton (1945). The carbon extracted from the culture filtrate by CHCl_3 represented less than half of the non volatile carbon of the metabolic products. The carbon accounted for by the four known metabolic products, i.e. 81 mg/l of the culture filtrate, was small compared with the total CHCl_3 soluble carbon of the filtrate (mean for the four strains, 792 mg/l) and it is clear that the greater part of the soluble metabolic products of the fungus remain to be identified.

The carbon balance of a culture of *T. roseum* (*Cephalothecium roseum* Corda) was determined by Birkinshaw, Charles, Hetherington & Raistrick (1931). It was shown that 'this species grew well on Czapek Dox solution, but was only able to metabolize very slowly the glucose supplied, since, even after 67 days, only about 65% of the glucose had been utilized'. The fungus was shown to have a respiration coefficient of 1.19.

Properties of the lipids

The CHCl_3 extract of the mycelium consisted mainly of a liquid mixture of esters, which amounted to 20% of the weight of the dry mycelium. The crude oil, dark brown in colour, was decolorized by passage through a column of charcoal. The almost colourless filtrate was liquid at 20° and crystallized partially below 15°. It had the following constants: saponification value, 194; iodine value, 57.

After saponification, the fatty acids were separated into saturated and unsaturated fractions by the lead salt method (Twitchell, 1921). The mixed fatty acids (151 g) yielded 47 g (31%) of saturated and 95 g (63%) of unsaturated acids. The latter contained a small proportion of linoleic acid, but no linolenic acid was detected. The major component was probably oleic acid. A portion of the unsaturated acids (0.5 g) was dissolved in CHCl_3 and excess bromine added. When the reaction was complete, the excess bromine and CHCl_3 were distilled off. The residue was completely soluble in ether indicating the absence of linolenic acid. After distillation of the ether, the residue was dissolved in hot light petroleum (b.p. 60–80°). On cooling colourless needles of linoleic acid tetrabromide (0.05 g) separated. Recrystallization from light petroleum gave soft needles, m.p. 115° (literature value m.p. 114–115°).

Table 1. *Partial carbon balance of metabolic products of four strains of Trichothecium roseum*

Strain no.	Initial glucose carbon (g/100 ml.)	Period incubation (days)	Residual glucose carbon (g/100 ml.)	Total carbon in solution (g/100 ml.)	Carbon in volatile neutral compounds (g/100 ml.)	Carbon accounted for by difference (as % of original glucose carbon)	Carbon extracted from culture filtrate by CHCl_3 (g/100 ml.)	CHCl_3 extracted carbon (% of original glucose carbon)	Mycelium dry weight (g)	Carbon content of mycelium (%)	Mycelial carbon (% of original carbon)	Fungistatic activity as trichothecin after incubation for 42 days (mg/l.)	Final pH at time of harvest
109	1.317	58	0.012	0.169	0.009	11.24	0.070	5.31	4.81	49.89	18.2	4.5	7.2
198	1.317	71	0.044	0.194	0.006	10.93	0.029	2.20	3.87	53.77	16.8	27.1	7.1
227	1.317	42	0.056	0.153	0.006	6.90	0.022	1.67	4.38	51.17	17.0	28.9	7.8
271	1.317	42	0.024	0.178	0.002	14.53	0.088	6.68	3.14	48.54	11.6	18.7	8.9

SUMMARY

1 The isolation and properties of roseins I and II from the mycelium of *Trichothecium roseum* and of rosein III from the culture filtrate are described

2 Rosein I, colourless tetrahedra, m p 210° , $[\alpha]_D^{24} - 112.5$ in chloroform (c, 1) has the molecular formula $C_{15}H_{22}O_3$. It is a neutral ketone, which reacted with one equivalent of hot ethanolic alkali and yielded a lactone, isorosein I, m p 144° , on acidification. The latter has an hydroxyl but no ketonic group. On oxidation with potassium permanganate, rosein I gave a monobasic acid, $C_{16}H_{24}O_4$, m p 242° .

3 Rosein II, $C_{16}H_{22}O_3$, crystallized in colourless fibrous needles, m p 186° , $[\alpha]_D^{25} + 5.9^{\circ}$ in chloroform (c, 2). It has no ketonic or hydroxyl groups, but its properties correspond to those of a lactone. Oxidation with potassium permanganate gave a monobasic acid, $C_{16}H_{22}O_5$, m p 296° .

4 Rosein III, $C_{20}H_{28}O_4$, crystallized from toluene in colourless plates, m p 221° , $[\alpha]_D^{19} - 124^{\circ}$ in chloroform (c, 1). It was a neutral ketone which, on

treatment with hot alkali, yielded isorosein III, m p 155° .

5 Rosein II inhibited growth of *Bacillus subtilis* at 5 p.p.m. It was slightly active against *Mycobacterium tuberculosis*, inhibited *Myco. phlei* at 1 in 81,000 and had no effect on *Myco. butyricum* at 1 in 1000. Roseins I and III had no effect on three test bacteria and on *Penicillium digitatum*.

6 The bitter principle of *Trichothecium roseum* observed by Iwanoff (1904), and others has been shown to contain trichothecin.

One of us (S.E.M.) wishes to acknowledge a grant from the Therapeutic Research Corporation of Great Britain Ltd., by which the work at the London School of Hygiene and Tropical Medicine was supported. The authors are grateful to Prof H. Raistrick, F.R.S., for his interest and encouragement.

Note added in proof. A paper has recently appeared by Robertson, A., Smithies, W.R. & Tittensor, E. (*J. chem. Soc.* 1949, p. 879) describing the isolation of rosenonolactone and rosonolactone from *Trichothecium roseum* Link. It appears probable that these two substances are identical with roseins I and II respectively.

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Acid-soluble Pigments of Molluscan Shells

2. PIGMENTS OTHER THAN PORPHYRINS

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(Received 18 November 1948)

The object of the study described in this paper was to identify, if possible, the acid-soluble shell pigments which accompany shell porphyrins in several groups of marine molluscs (Comfort, 1948, 1949a) or which occur in related genera, and to examine their chromatographic behaviour and phylogenetic distribution. Work was concentrated on the shell pigments of the bivalve genus *Pinctada*, in the hope of relating them to the metabolism of porphyrins. This paper contains a summary of their chromatographic behaviour and general properties, compared with those of other acid-soluble molluscan shell pigments.

Previous work on shell pigments contains descriptions of a number of substances, but no clear picture of their chemical and phylogenetic relationships. Linear tetrapyrroles are known to occur in shells (Krukenberg, 1883; Schultz, 1904). Tixier (1947) isolated a number of fractions by chromatography of acid-methanol extracts of *Turbo regenfussi* and *T. marmoratus*, and identified the main crystallizable shell pigment as a glaucobilin (Turboglucobilin). Dhéré & Baumeler (1929) regarded the red pigment of *Halotis refuscens* as a pyrrole derivative. The blue pigment of *H. californiensis* has been variously described as an indigoid

and as a tetrapyrrole (Krukenberg, 1883, Schultz, 1904, 1931, Kodzuka, 1921, Schultz & Becker, 1931, Lemberg, 1931), its investigation will form the subject of a separate paper. The pigments described below appear to be distinct both from the known indigoids and from the bile pigments

EXPERIMENTAL

Extraction Coarsely broken shell was macerated for 12–24 hr at room temperature with conc HCl, or with 33% (v/v) aqueous H_3PO_4 (sp gr 1.75 diluted 1 in 3). Octanol was added to prevent frothing. When extraction was complete, the coloured solutions were filtered through cellulose wool, and the pigments separated by chromatography.

Chromatography This was carried out directly from aqueous acid solution, with talc as the adsorbent. Talc of normal pharmaceutical quality was shaken with 3N HCl and poured through a funnel containing glass beads into tubes, the lower ends of which were plugged with cotton wool supported on hollow ceramic insulators. The columns were then packed by suction, with frequent tapping to expel bubbles.

The solutions for chromatography were adjusted by dilution to an acidity of N to 2.5N HCl, and passed through the columns under suction. Under these conditions the porphyrins tend to form a sharp zone, detectable by ultra-violet fluorescence, close to the top of the column. The concomitant pigments falling below this zone were eluted with increasing strengths of acetone in 2N HCl or H_3PO_4 .

The adsorptive power of different samples of talc varied, and results given in this paper refer to a single batch of known behaviour. Increase in the acidity of the solvent above 2N HCl produced blurring of the zones, and, with some extracts, reversal of the order of adsorption.

Spectroscopy Visible absorption bands were measured with a Hartbridge reversion spectroscope. All wave lengths are stated in $m\mu$.

RESULTS

Distribution

The distribution of non porphyrin acid soluble shell pigments closely follows that of the porphyrins. In general, the pigments of higher molluscs cannot be separated from the conchiolin of the shell. They appear to be chromoproteins. Absorption spectra obtained by trans illuminating the shell give no clue to the nature of the chromophore.

Acid soluble pigments occur in, and account for the colour of, most archaeogastropod shells, the pearl mussels, the purple Anomidae such as *Placuna* and the tectibranch opisthobranchs. In the archaeogastropods their range is wider than that of the shell porphyrins, since they occur in the porphyrin free species of Trochidae, in the Turbinidae and in *Halotis*. The red pigment of recent *Pleurotomaria*, which could not be obtained for extraction on account of their rarity, is probably also acid soluble.

Comparison of chromatograms

The chromatograms of a number of pearl mussel shell extracts show a close general similarity. In several species the lowest major pigment band was pink, the second blue and the third violet. In these extracts the porphyrin fraction, varying in colour from violet to brown, appeared higher on the column than the non porphyrin pigments.

Preliminary study of the chromatograms of extracts of archaeogastropod shells shows a greater diversity of patterns. Chromatograms of species of *Fissurella*, *Trochus*, and *Clanculus*, contained pigment bands similar in appearance and sequence to those of the bivalves, but lack of material prevented further study of these forms.

In all forms, quantitative variation in the bands was marked, and the chromatograms of pooled extracts differed from those of single specimens. This variation was particularly marked in *Pinctada vulgaris*.

In the following protocols, typical chromatograms of several forms are described in detail. The species of *Halotis* and *Turbo* are included for comparison with the bivalves, since they contain pigments which have been studied by other workers.

CHROMATOGRAPHY OF SHELL EXTRACTS

Pinctada vulgaris

Six valves were broken and macerated overnight in a mixture of 170 ml H_3PO_4 (sp gr 1.75) and 430 ml. water. The port-wine coloured extract was filtered and chromatographed on a column of talc, 20 × 3 cm. The undeveloped chromatogram showed the following bands (Fig. 1).

Band no	(Top)	Width (mm)
	Yellow	3
	Grey	3
	Ochre	3
5	Brown	6
4	Pink	2
3	Blue	6
2	Pink	15
	Blank	6
1	Purple	3 moving down

Band 1 was eluted by further addition of solvent. Bands 2 and 4 were removed by approx 3% (v/v) acetone in 33% aqueous H_3PO_4 as a single pink, non fluorescent fraction (absorption bands 545, 497 $m\mu$). Band 3 moving down divided into two, a lower purple and an upper indigo blue (3a), both showing pink fluorescence. The fraction containing these bands gave absorption maxima at 593, 495, 465 $m\mu$ in N HCl. The brown band containing most of the porphyrin, accompanied by a non fluorescent brown band, and another non fluorescent purple band, not separable on the column, was eluted with 30% (v/v) acetone in acid, the mixed eluate giving absorption bands at 593, 572, 550 (intense), 491.5 (faint), 467 $m\mu$.

Rechromatography of porphyrin fractions The porphyrin fraction was warmed and the acetone removed by a current

of air. The solution was passed through a talc column 10×0.75 cm, giving four zones, from above yellow, pink (strong fluorescence), yellow, blue green. The two lower bands were removed by development with 1% pyridine in 33% H_3PO_4 , and the remainder by 10% (v/v) aqueous pyridine. The second eluate was diluted with twice its volume of water and again chromatographed, giving a wide pink zone. Traces of green pigment passed out in the solvent. The column was developed with aqueous acetone (1 part in 6 parts of water)

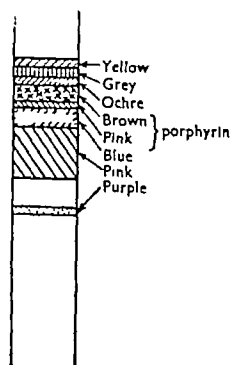


Fig 1 Chromatogram on talc of acid extract of *Pinclada vulgaris* shells (crude extract, see p 200)

A wide porphyrin band passed down, leaving another, equally fluorescent but pinker, to be removed by 90% aqueous acetone. The spectra of these fractions differed (Table 1) the spectra became identical on addition of excess 3N HCl: upper band—624, (606.5), 590–568, 535, 490 $m\mu$, lower band—567, 541, 504 $m\mu$. On addition of conc HCl to both fractions, the spectra became identical 594, 574, 550 $m\mu$. Both solutions were clear violet in colour.

Rechromatography of non-porphyrin fractions Fraction 1 was adsorbed on a small (10×1 cm) column, and eluted with acetone as a brownish purple solution, in which no band could be detected visually.

Fraction 2, though not fully homogenous on re-adsorption, could not be further resolved on talc. Its spectral absorption curve is shown in Fig 2.

Fraction 3–3a, which was manifestly complex on the first column, was diluted and rechromatographed on a 10×1 cm column. It gave a greyish upper zone, a purple band and a narrow indigo blue zone, and on further development with acid acetone (approx 1 part acetone + 9 parts of 20% (v/v) aqueous H_3PO_4), the purple band could be seen to overlap a wider blue zone which overlapped it on each side, but travelled down with it. The purple band had now a very strong red fluorescence of typical porphyrin appearance, but this was evident only in adsorbed material, the eluate, a clear-cut, violet fraction, was non fluorescent both on isolation and after addition of 0.25 ml. conc HCl to about 3 ml of eluate. It showed strong bands at 507–498 $m\mu$ max. 500 $m\mu$, 608–578 $m\mu$ max 602 $m\mu$.

The column was now washed down with 50% acetone in 20% (v/v) H_3PO_4 , and a purple brown band was recovered. It showed a single faint adsorption band about 500 $m\mu$.

Subsequent extraction and working up of large amounts of material especially re-extracts and dregs of spent shell, gave

larger proportions of the 3–3a fraction, having a strong fluorescence both on the column and in solution. Spectroscopy of these fractions in the ultraviolet showed no Soret

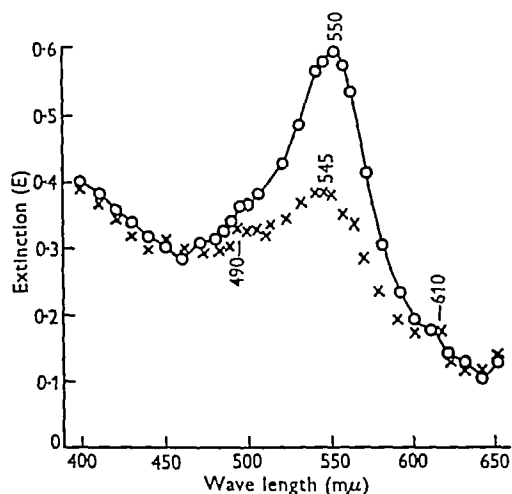


Fig 2 Spectral absorption (in N HCl acetone) of lower pigment fractions from chromatograms of shell extracts (Beckmann spectrophotometer) ○—○—○, *Pinclada vulgaris*, ×—×—×, *Malleus regula*

band at a cell depth of 0.25 cm, but a trace ($\lambda = 405 m\mu$) at 1 cm, insufficient to account for the colour of the fraction. The inequality of these results and the lack of relation between colour and fluorescence in successive specimens suggest that the porphyrin component is distinct from the main 3–3a pigment.

Preparation of pigment fractions In the isolation of larger quantities of material from *P. vulgaris* a somewhat different

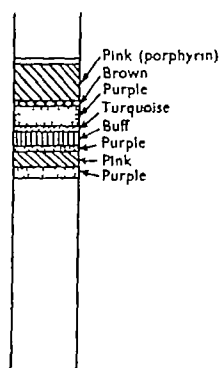


Fig 3 Chromatogram on talc of acid extract of *Pinclada vulgaris* shells (partially purified, see text)

technique was used. Coarsely ground shells (500 g) were extracted successively with 500 ml 50% (v/v) H_3PO_4 , acetone and 3N HCl. The acetone was removed, and all extracts pooled, then diluted and allowed to percolate under suction through 1 kg of pure talc in a large, squat column (20×10 cm). The pigments were eluted with acetone. Zone 1 was rejected in the filtrate from the percolation. The

acetone eluate was now shaken with sufficient xylene to cause phase separation, the main pigments passing into the aqueous layer. A small amount of porphyrin passing into the xylene acetone layer was recovered separately. The water phase was now concentrated under reduced pressure and placed on a column of talc, 12 x 3 cm. The resulting chromatogram was highly complex.

(Top)	Width of band (mm)
Debris ochre	
Blank	2
(Pink red fluorescence)	12
Brown	1
Purple	8
Turquoise	1
Buff	4
Violet	0.5
Pink	5
Violet	2

The column was developed with 15% (v/v) acetone in *N* HCl. A brown and a pink band passed down (fraction A). The column was then extruded and cut. The violet and blue middle section, the brown layer above it and the top pink band of porphyrin were taken up separately in acetone and filtered (fractions B, C and D). They were left to evaporate at room temperature.

Fraction A on rechromatography gave an ill defined separation into a lower brown and an upper pink band, probably identical with zone 2 of the initial chromatogram. Fraction B gave four zones, from above, violet, turquoise, unhomogeneous violet-brown, and a yellow trace, possibly extraneous, which washed out with 0.5*N* HCl. On cutting the column and redissolving in acetone, these fractions all showed bands at 587–556, 511–493 *mμ*, which were strongest in the violet top fraction. The lowest mixed band had an additional maximum at 465 *mμ*.

Fraction D was concentrated and dried *in vacuo*. The dry residue was extracted with ethyl acetate, and about a third of the porphyrin, estimated by fluorescence, passed into solution (Soret band *N* HCl, 405.5 *mμ*, 3*N* HCl, 407.0 *mμ*), and was extracted by shaking with *N* HCl. The remaining ethyl acetate layer was now brown, with bands at 500, 480, and a trace near to 550 *mμ*. It was non fluorescent. The residues of the original extract were treated with 3*N* HCl, and the remaining porphyrin passed into solution. A black insoluble residue remained on the filter. The two fractions of porphyrin from this rough separation were rechromatographed on paper by the technique of Nicholas & Rimington (1949), and are reported upon separately (Nicholas & Comfort, 1949).

The turquoise, red fluorescing fraction (3a of initial chromatogram) was rapidly converted on standing to a yellowish, ether soluble material, which could not be wholly

re extracted from acetone-ether mixture by shaking with 3*N* HCl. This yellow material was non fluorescent and showed only a faint band close to 500 *mμ*.

The spectral characters of *Pinctada* fractions are summarized in Table 1.

All total extracts of *P. vulgaris* contained a brown, xylene and amyl alcohol soluble material which could be removed by partition from the acid solution.

Malleus regula Lk

About one quarter of one valve, including the area of hinge where traces of red fluorescence could be seen, was extracted with 100 ml 2*N* HCl. Extracts of all parts of this shell were fluorescent, although fluorescence is not general in the intact shell.

Chromatography The conspicuous blue purple complex of *Pinctada* (bands 3–3a) was absent, but the chromatogram was otherwise similar. Band 1 contained more material than in *Pinctada*, but was identical in appearance and behaviour. The pink band (2) accounted for most of the visible colour, the porphyrin remaining as a sand coloured zone at the surface of the talc, within the next (pink) band.

On development with 40% acetone to remove band 2, a brown fraction passed down and through it, appearing in the eluate just before the lower border of band 2. This fraction was distinct from the main body of band 2, and different in colour. The porphyrin band was small and rather faint.

Pinctada margaritifera

Acid extracts in HCl are reddish brown, with little or no porphyrin fluorescence. The olive shell becomes purple on contact with the acid. Large amounts of pigment can be recovered from the acid treated shell by rewashing with acetone. The acid fraction showed bands at 501, 465.5 *mμ*, and the acetone fraction at 503 *mμ*, but when the acetone was removed by shaking with xylene and light petroleum, and the hypophase treated with excess 2*N* HCl, the spectra were identical. Another shell gave a different acid spectrum, the pigment being olive instead of reddish, and having bands at 481–513 and 555 *mμ*. Addition of a few drops conc HNO₃ produced a shift to 464 and 504 *mμ*. The fractions were almost homogeneous on chromatography, except for a very small upper porphyrin zone.

Placuna sella

A deeply coloured shell was selected. Chromatography of the phosphoric extract was more difficult than in *Pinctada*, owing to the presence of brown bands overlapping the general pattern. The bands from above were porphyrin (olive), green, violet, pink, olive, violet, brick, broad pink. The

Table 1. Characteristics of fractions obtained by chromatography on talc of crude extracts in phosphoric acid of shells of *Pinctada vulgaris*

Fraction	Crude extract	1	2	3	4 and 5
Eluent	—	H ₃ PO ₄ (33% v/v)	Acetone* (3% v/v)	Acetone* (10% v/v)	Acetone* (30% v/v)
Colour of eluate	Wine	Purple	Pink	Indigo violet	Pink
Abs maxima (mμ)	595, 553, 464	(500?) Very weak	545, 498	602, 500	614, 574, 551, 492, 464
Remarks	—	Single zone	Three zones eluted together	Two zones eluted together	Porphyrin homogeneous

* Dilutions of acetone made in 33% (v/v) aqueous H₃PO₄.

lowest fraction on elution with 30% acetone was not homogeneous. It showed a strong double band at 493.5 and 466 m μ . The next fraction (eluted by 50% (v/v) acetone in 33% phosphoric acid) had a single band (480–505 max 491.5 m μ). With higher concentrations of acetone a further purplish pink band appeared high on the column (490–506, 553–587 m μ) and moved down, followed by the porphyrin band. No clear-cut separation could be achieved on rechromatography. The lowest pigment was in all respects similar to the acid fraction of *P. margaritifera*, an entirely unrelated form.

Halotis madae

The pigment is most easily extracted from immature shells. It is present in the outer layer of the shell, and also in the substance of the protoconch.

Shells extracted with conc. HCl give a port-wine coloured solution, changing to brown on dilution. The red solution shows bands at 560–530 and 472–455 m μ . On standing, it turns purple (max 544, 495 m μ). The Ehrlich (diazotization) and Gmelin reactions are negative, and the pigments cannot be transferred to ether from neutral solution.

Chromatography of the acid extract on talc gives two main zones, and a yellow filtrate. These are red (below) and blue (above); the red zone becomes straw coloured in acetone, but the red is restored by the addition of HCl. Only the red fraction shows an extremely faint red fluorescence; the blue pigment is non fluorescent. It does not give a colour reaction with NaNO₂. No further colour change occurs with FeCl₃. On neutralizing total extracts and shaking with CHCl₃, however, traces of Lemberg's (1931) blue pigment can be extracted. The identity of the *Halotis* pigments is discussed in a separate paper (Comfort, 1949b).

Turbo marmoratus

The main pigment of this species has been crystallized and fully investigated by Tixier (1947) under the name of turboglaucobilin. It is a blue, non fluorescent bilatriene having a visible and an ultraviolet spectrum similar to those of glaucobilin. It was isolated for comparison with the talc chromatograms already obtained. Two specimens of *T. marmoratus*, which is probably identical with the *T. regenfussii* studied by Tixier, and which is named by him as a source of an identical pigment, were broken and extracted separately, half with acid ethanol (20% conc. HCl in ethanol) the pigments being transferred to CHCl₃ by the method employed by Tixier (1947), and half with 5N HCl for talc chromatography.

The direct chromatogram of the HCl extract gave three zones (from above, blue, violet, brown) corresponding to those found by Tixier (1947) on alumina from CHCl₃. Porphyrin was not detected. Solutions of rechromatographed turboglaucobilin ethyl ester in CHCl₃ were non fluorescent in ultraviolet light.

Mixed chromatography

An attempt was made to relate the pigments of the *Pinctada* series to the known substances *Halotis* blue and turboglaucobilin by chromatography of mixtures. In the mixed chromatogram of extracts of *Turbo*, *Halotis crachei* and *Pinctada*, no evidence of identity was obtained; the pigments of the *P. vulgaris* series and the lower violet

and brown bands of *Turbo* falling below the porphyrin zone, and *Halotis* blue, turboglaucobilin, and the CHCl₃ soluble pigment of *P. margaritifera* above it (Fig. 4). Turboglaucobilin is more easily eluted by acetone than the porphyrin zone, and passes through it. Mixed chromatograms of *Pinctada*

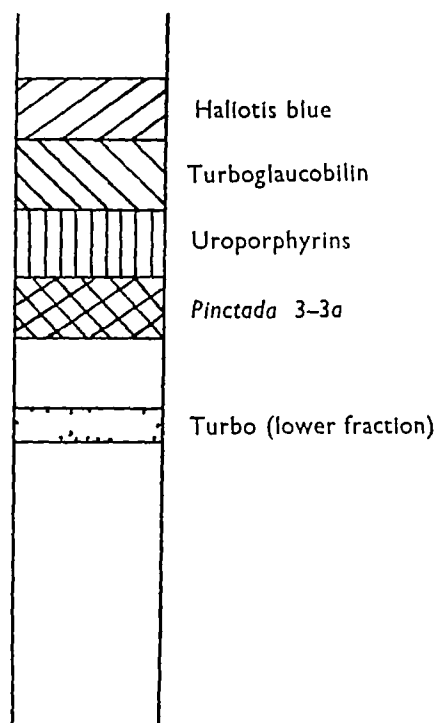


Fig. 4 Chromatogram (talc, N HCl) of artificial mixture of shell pigments

pigments with acid solutions of the products of the Gmelin reaction performed with conc. HNO₃ upon turboglaucobilin and upon biliverdin from cat bile showed no coincidence of zones. A solution in CHCl₃ of the products of this reaction upon turboglaucobilin was, however, spectrally similar to the CHCl₃-soluble fraction derived from *P. margaritifera*.

DISCUSSION

The pearl mussel pigments, especially those of *Pinctada* and *Mallemus*, can be separated into a number of fractions which show a superficially similar chemical behaviour. The number of substances present, the differences between related species and among individual shells of the same species and the insolubility of the pigments in organic solvents makes the purification and crystallization of individual substances extremely difficult, and pure specimens were not isolated. Except in the case of *Halotis* blue, which will be discussed in a separate paper (Comfort, 1949b), the identity of these pigments has not been established. The *Pinctada* family of pigments exhibits no definite colour reaction comparable to that given by *Halotis*

blue with nitrites (Schultz, 1931) This group does, however, behave as a consistent chromatographic series

A rather similar family of pigments occurs in several archaeogastropod shells, especially among the red Trochidae and Fissurellidae, though chromatograms of these forms could not be repeated to our satisfaction owing to shortage of material In other forms related to the porphyrin-producing groups, such as *Turbo* and *Halotis*, we have been able to confirm Krukenberg's (1883) finding of bilitrienes but bilitrienes and porphyrins do not appear to coexist

Mixed chromatography of *Pinctada* extracts with turboglucobilin, with *Halotis* blue and with the products of an incomplete Gmelin reaction performed on biliverdin from bile, shows no evidence of correspondence between known and unknown pigments

The acid soluble pigments of *Pinctada* have the following characteristics they are indicators, changing to grey or greenish blue in alkaline solution, they are adsorbed from acid solution on talc in a consistent order, red falling lowest and violet highest upon the column, they are insoluble in organic solvents except acetone, ethanol and methanol, but they pass into amyl alcohol from acid solution on prolonged standing in a manner

which resembles the behaviour of porphyrins, and strongly suggests esterification, they are non fluorescent, and their visible absorption spectra are characteristic

A close phylogenetic relationship exists between these pigments and shell porphyrins They are found in typical purplish or reddish colour schemes, which seem to occur most commonly in primitive or unspecialized forms

SUMMARY

1 The distribution of acid soluble pigments in shells has been found to resemble that of the porphyrins

2 The pigments can be separated by chromatography from aqueous acid upon talc A characteristic series of pigments, which have not been identified, occurs in several bivalves and probably also in the Trochidae and Fissurellidae Linear tetrapyrroles occur in the Turbidae, where porphyrins are absent

3 The pigments of higher molluscan shells are not extracted by acid, and appear to be chromoproteins The possession of acid soluble shell pigments is characteristic of the archaeogastropods, the lower bivalves, and the tectibranch opisthobranchs

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Acid-soluble Pigments of Molluscan Shells

3 THE INDIGOID CHARACTER OF THE BLUE PIGMENT OF *HALIOTIS CRACHERODII* LEACH

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(Received 18 November 1948)

The investigation described in this paper was designed to establish the identity of the blue shell pigment of the abalone (*Halotis cracherodii*), and to examine its relationship with other acid soluble shell pigments

The pigment was described by Krukenberg (1883), and has received considerable attention (Schultz, 1904, 1931, Kodzuka, 1921, Schultz & Becker, 1931,

Lemberg, 1931) Workers have been divided between the later view of Schultz (1931) that it is an indigoid body, and that of Schultz (1904), Lemberg, Kodzuka and Krukenberg that it is pyrrolic

Lemberg (1931) described the extraction of the pigment with acid ethanol The extract was treated with chloroform followed by 0.05N HCl, which removed a blue material (B), leaving a green

chloroform solution (A) Similar methods of extraction were used by other workers In no case was the extract chromatographed Although failing to behave as a glaucobilin (Lemberg, 1931) the blue pigment gave the pine splinter reaction for pyrrole (Kodzuka, 1921) and a substance resembling a di pyrlylmethene on treatment with phosphonium iodide (Lemberg, 1931) The literature is reviewed by Lederer (1940)

EXPERIMENTAL AND RESULTS

Identity of material The species figuring in the literature as *Halotis californiensis* Sow is almost certainly *H. cracherodii* Leach, of which *H. californiensis* is an uncommon variety The results of Kodzuka (1921) refer to *H. gigas* In the present study shells of *H. cracherodii* from California were used throughout

Chromatography of the pigments of *Halotis cracherodii*

Extraction Extracts were prepared from coarsely broken shell (a) with acid ethanol, for the preparation of Lemberg's (1931) A and B fractions, (b) with 5N HCl, for direct chromatography on talc by the method developed for the isolation of porphyrins (Comfort, 1949 a), (c) by preliminary acid extraction, the pigment being concentrated by shaking with amyl alcohol, into which it passes from aqueous acid

(a) When shells are extracted with acid ethanol, and the extract partitioned according to the instructions of Lemberg (1931) his A and B fractions are readily prepared The A fraction, when further treated with conc HCl, is removed almost completely from the CHCl_3 phase as a deep purple material showing bands at 661–586, 539 and 461 m μ . When chromatographed on talc, this extract gives a green blue upper and a violet lower zone

(b) Since our object was to compare the pigments of this species with those accompanying porphyrins in other forms, especially *Pinctada*, most of our extracts were made in 5N HCl and chromatographed upon talc from aqueous acid, using acetone HCl or 0.5N NaOH as developer

A medium sized specimen (length 13 cm) was coarsely broken, and as much as possible of the nacreous shell removed. It was macerated for 24 hr with 500 ml. 5N HCl, and the blue extract decanted from green insoluble residue A column of talc was packed from suspension in N HCl by suction over cotton wool in a tube 20 x 2 cm The extract was filtered and drawn through the column

The bands of the undeveloped chromatogram are shown in Fig 1a The lower four fractions, eluted together by 10% (v/v) acetone in N HCl, were shaken with amyl alcohol, which removed orange and yellow pigments having a positive Gmelin reaction, and left a green, Gmelin negative hypophase The upper blue and purple bands, eluted slowly by acid ethanol (20% HCl (v/v) in ethanol), gave the intense red colour with NaNO_2 which characterizes *Halotis* blue No ultraviolet fluorescence was detected in this chromatogram

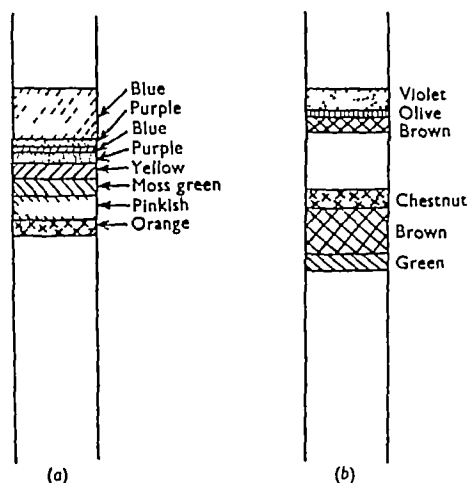


Fig 1 (a) Undeveloped chromatogram on talc of acid extract of shell of *H. cracherodii* (b) Partly developed chromatogram on alumina of ether soluble fraction of *H. cracherodii* extract

(c) A similar acid extract was prepared (see flow sheet, Fig 2) by macerating two large shells with 300 ml. 7.5N HCl The deep blue solution was filtered and shaken with 600 ml amyl alcohol in small successive portions The amyl alcohol solution, containing most of the pigment, was evaporated to dryness under reduced pressure, and the residue taken up in 20 ml ethanol. Ether (20 ml.) was added, the mixture was placed overnight in the refrigerator, and then treated with excess 2N HCl The blue pigment remained in the hypophase, leaving a greenish yellow ether layer

The ether solution was chromatographed on a column (prepared from a suspension in light petroleum) of alumina (Spence '0') in a tube 10 x 1 cm. The column was washed

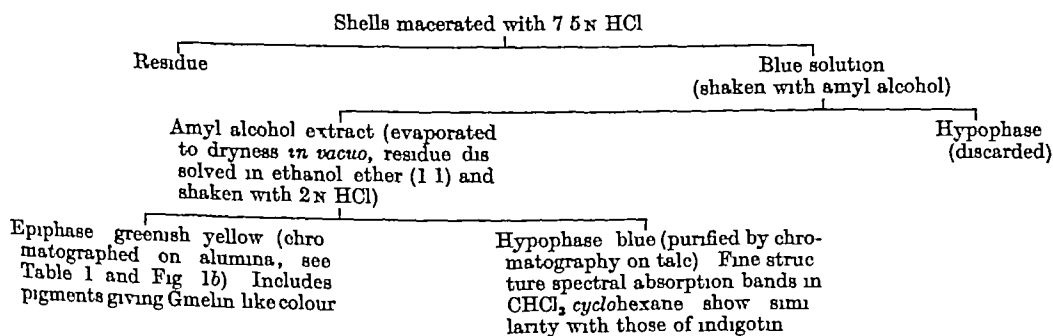


Fig 2 Flow sheet to illustrate separation of pigments from *Halotis cracherodii*

with several changes of ether, and showed the zones listed in Table 1 from above downward. Development with acetone 40% (v/v) in ether produced the chromatogram shown in Fig. 1b. The violet upper band was found to consist of the alkaline phase of the main blue pigment. The fractions eluted by acetone gave a positive Gmelin reaction.

Table 1 *Chromatogram on alumina of ether-soluble pigments from crude extract of* *Halotis cracherodu*

Colour of zone	Width (mm)
Moss green	3
Brick red	3
Orange	10
Buff	7

The acid alcohol solution (hypophase of the initial ether partition) was freed of amyl alcohol by partial evaporation, and proved to be almost homogeneous on talc chromatography. This fraction was eluted with acid ethanol, again transferred to amyl alcohol and evaporated to dryness under reduced pressure. The residue was examined spectroscopically.

Ultraviolet spectroscopy and indigoid nature of Halotis blue

The rechromatographed material described above was dissolved in CHCl_3 -cyclohexane, and its fine structure bands compared with those of the known indigos by means of a Hilger quartz spectrograph. The results are given in Table 2.

Table 2 *Ultraviolet absorptions of Halotis blue and of indigotin in chloroform-cyclohexane solution*

Absorption maxima (A)	
<i>Halotis</i> pigment	Indigotin
3570	3560
3370	3370
3226	3210
3070	3070
—	2856
—	2745

The similarity of these spectra affords strong evidence for the identity of the chromophoric groups in the two substances, and the conclusion appears justified that the *Halotis* pigment contains an indigo closely related to, but not identical with, indigotin. The objections of Lemberg (1931), based on solubility considerations, do not contradict this conclusion although they suggest that the substance is not one of the known indigo pigments. Its solubility in acids is probably due to the presence of side chains attached to the indigo nucleus.

DISCUSSION

We have shown (Comfort, 1948, 1949a, b) that porphyrins are widely distributed among the Archaeogastropoda, but almost wholly absent from the species of *Halotis* which we have examined. If *Halotis* blue is a pyrrole derivative, as suggested by Lemberg (1931) and Kodzuka (1921), it might be held to represent an end product of the pigment

cycle which gives rise to porphyrins in the Trochidae (Tixier, 1945), and to biliterenes in the Turbinidae (Krukenberg, 1883; Tixier, 1947). If, on the other hand, Schultz (1931) was correct in regarding it as an indigoid body, it is the only substance of this kind known to occur in molluscs other than the Stenoglossa, many of which produce 6,6'-dibromoindigotin as a visceral and egg pigment.

Lemberg's objections to the findings of Schultz are threefold: that the pigment does not show the typical solubilities of indigo blue itself, that it is more basic than any known indigoid, including the dialkyl indigos, and that resemblances in the visible spectrum are not conclusive evidence of relationship between this body and the spectrally similar indigos cited by Schultz (*O*-methylindigo 620, dibromoindigotin 623 μ , Kruss & Oeconomides, 1883).

The red material produced from *Halotis* blue by nitrites in acid media bears some resemblance to that produced from bile pigments in the Gmelin reaction. Schultz (1931) regards the red colour as being due to isatin. When nitrite is added to a solution of *Halotis* blue purified by chromatography, the development of the red colour requires a considerable excess of hydrochloric acid. With nitric acid the colour change is rapid, but not typical of the Gmelin reaction. The pigment becomes first violet and then red, with disappearance of the band at 622 μ and appearance of two new bands close to 500 and 550 μ . Other fractions from the chromatogram show the characteristic sequence of colours given by bile pigments, but none produces a colour comparable with that of the nitrite reaction. The red material is very soluble in amyl alcohol, and only slightly soluble in chloroform. Its spectrum does not coincide with that of the Gmelin products, or of the purplish red solution produced by treating turboglucobilin (Tixier, 1947) with nitric acid. Moreover, a similar colour appears spontaneously in acid solutions on standing, or very rapidly on the addition of hydrogen peroxide, in the presence of which the colour finally fades to pinkish orange.

The colour change of the *Halotis* pigments in this case does not, therefore, coincide with that given by biliterenes. It is far closer to the reaction described by Nencki & Sieber (1882) in the uroresin test. Herter (1908), Homer (1915), Watson (1938, 1939), Waldenström (1937) and Rimington, Holiday & Joje (1946) have discussed this test in some detail. Three main red pigments have been isolated from porphyrin-free urines: uroresin, indirubin (Watson, 1938, 1939, Rimington *et al.* 1946) and urocarmine (Fearon & Thompson, 1930). The solubilities of these pigments, and their spectral absorption bands, are compared in Table 3.

The end product of the action of sodium nitrite on *Halotis* blue resembles uroresin both in these

Table 3 Comparison of absorption spectra of urorosein and of the red derivative of *Halotis blue*

Substance	Solvent	Spectrum (abs max $m\mu$)	Solubility	
			Chloroform	Amyl alcohol
<i>Halotis</i> derivative	Amyl alcohol, conc soln	565-468	Slight	Marked
	Amyl alcohol, dilute soln	522-492	—	—
	2N HCl, conc soln	559-550	—	—
	2N HCl, dilute soln	540-490	—	—
Urorosein (Watson, 1939)	Amyl alcohol	555-557	—	Marked
	2 N HCl	544-511	—	—
	From indolylacetic acid (Homer, 1915)	552-531	—	—
		505-492	—	—
From indolylaceturic acid (Homer, 1915)		579-539	—	—
		502-485	—	—
		No data	—	Marked
Urocarmine (Fearon & Thompson, 1930)				

properties and in being reversibly decolorized by sodium acetate. Studies with the Beckman spectrophotometer gave density curves which varied somewhat from specimen to specimen, that given in Fig 3 being typical. In acid solutions the curve closely resembles that of urorosein (Rimington *et al* 1946), which is generally admitted to be an indole derivative.

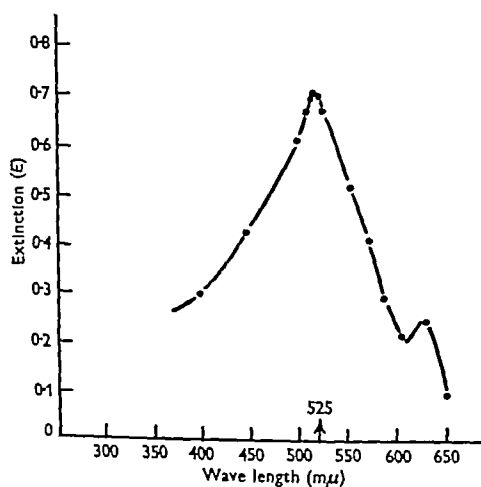


Fig 3 Spectral absorption of red derivative (produced by addition of HNO_2) of *Halotis* blue in acetone

In the present paper, a comparison is recorded of the fine structure absorption in the ultraviolet of purified *Halotis* blue and indigotin. The similarity observed is so marked as to justify the conclusion that *Halotis* blue possesses an indigoid structure. The colour reactions observed with nitric and nitrous acids are reconcilable with this conclusion. The solubility of the *Halotis* pigment in dilute hydrochloric acid indicates, however, that it must possess substituent groups modifying the character of the nucleus.

SUMMARY

1 The blue pigment of the shells of *Halotis cracherodii* has been purified chromatographically. It has an ultraviolet absorption spectrum almost identical with that of indigotin, but differs from that substance in its solubility characteristics.

2 The reaction of the blue pigment with oxidizing agents results in a product which resembles urorosein in its spectral and chemical properties.

3 *Halotis* blue is considered to possess an indigoid structure modified by the presence of substituent groups.

4 Other fractions derived from the same species contain pigments giving a reaction closely similar to the Gmelin reaction for bilitrienes.

NOTE

Added 16 May 1949

Since the publication of the preliminary results of this study, a fuller investigation of *Halotis* blue has been made by Tixier & Lederer (1949), who have kindly made their findings available to us in manuscript. Their figures for the molecular weight and elementary composition of *Halotis* blue methyl ester, purified by chromatography, are incompatible with an indigoid structure, and suggest a copromesobilviolins. They regard the pigment as a bilitriene differing from the known pigments of this type in its spectral characters and in the fact that its zinc complex is non fluorescent. A joint investigation of the interpretation of our findings and those of Tixier & Lederer will be undertaken, and its results published in a further communication.

Thanks are due to Prof C Rimington for the use of the Beckmann spectrophotometer and for access to unpublished work by him, and to Dr E. M. Jope, London Hospital Spectrographic Unit, Medical Research Council for the spectroscopic study of *Halotis* blue. The cost of the research was borne by the Yarrow Research Fund, London Hospital Medical College.

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Acid-soluble Pigments of Molluscan Shells

4 IDENTIFICATION OF SHELL PORPHYRINS WITH PARTICULAR REFERENCE TO CONCHOPORPHYRIN

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(Received 13 December 1948)

Particular interest has been attached to the nature of the porphyrins present in shells since Fischer & Jordan (1930) reported the occurrence in the marine mollusc *Pteria radiata* of a porphyrin, containing five carboxyl groups, which they named concho porphyrin, and which was the only pentacarboxylic porphyrin known in nature. Fischer & Holt (1934) later compared it with a synthetically prepared pentacarboxylic porphyrin. The findings of other workers (Fischer & Haarer, 1932; Waldenström, 1937; Tixier, 1945) indicate, however, that only uroporphyrins I and III and traces of coproporphyrin are responsible for the pink fluorescence of molluscan shells.

One of us (Comfort, 1949) has recorded an extensive survey of the pigments present in molluscan shells, and has noted the phylogenetic distribution of porphyrin.

Having at our disposal a method of separating porphyrins, by partition chromatography, into groups depending upon the number of carboxyl functions in the molecule (Nicholas & Rimington, 1949; Rimington, 1949), we have examined as many porphyrin containing shells of different species as possible, including *Pteria radiata* Lk, in the hope of confirming and extending Fischer & Jordan's (1930) observations. We have also had access to some of the original porphyrin material prepared by these authors (m.p. 270–273°) kindly provided by Prof

J Waldenström of Uppsala, but we have obtained no evidence for the existence of a pentacarboxylic porphyrin.

METHOD

Porphyrins and other acid soluble shell pigments are quantitatively adsorbed on talc, satisfactory separation of the individual porphyrins was not, however, achieved by this method. The acetic acid ether technique for the extraction of ether soluble porphyrins is difficult to apply to shell extracts on account of the large quantities of calcium salts.

Talc adsorption chromatography was used in order to separate the porphyrin as far as possible from the other shell pigments present, and finally the paper partition method (Nicholas & Rimington, 1949) for the separation of the individual groups of porphyrins.

Technique of talc chromatography

Preparations of columns. Talc powder (pharmaceutical) was shaken with excess of 2N HCl, and the suspension poured through a funnel containing some glass beads (to remove bubbles) into glass tubes 20 × 2 cm., the lower end of each being closed by a cotton wool plug resting on a ceramic insulator. The suspension was then packed with the aid of suction.

Extraction of shells. The shells were broken and decalcified for 24 hr in conc HCl, or in a mixture of phosphoric acid (sp gr 1.75) with an equal volume of water. The extracts were filtered through glass wool to remove debris, adjusted to contain between 1 and 2.5N acid by dilution with water, and poured into the tubes.

Chromatography The extracts were drawn through the columns under suction. The chromatograms were either developed with increasing strengths of acetone in aqueous 3N HCl, or dried for several hours over suction and extruded by air pressure from below, the fluorescent zones being cut out with a cover slip, or with a specially made glass knife.

Precautions The adsorptive power of various samples of talc showed considerable variation, and the acidity of the solvent should not exceed 2.5N, stronger acids lead to blurring of bands and reversal of their order. Packing from acid suspension was found necessary, even with 'acid washed' talc, to avoid cracking of the column when suction was applied.

The detailed appearance of the chromatograms and the spectral character of the non porphyrin bands has been described in a previous paper of this series (Comfort, 1949). The porphyrin present was localized to one narrow pink fluorescent band, except in the case of *Pteria vulgaris*, where division of the porphyrin was obtained during the separation of a blue pigment, spectroscopy of the porphyrin fractions in acid solution showed in each, however, a Soret band consistent with uroporphyrin ($\lambda = 405 \text{ m}\mu$ in 2N HCl).

Preparation of porphyrin for partition chromatography

The section of the talc columns containing the porphyrin, as shown by ultraviolet light, was cut out and placed in acetone containing 1% by volume of 3N HCl. This eluted all the porphyrin together with some of the other pigments adjacent to it on the original talc column. The eluate was filtered through a sintered glass funnel to remove the talc and the latter washed with HCl acetone solution. After combining the washings with the first eluate, the acetone was removed under reduced pressure at room temperature. The solution, which was highly fluorescent in ultraviolet light, but varied in colour with individual shells from brown to violet, was then passed through a small talc column to remove the remaining non porphyrin pigments and the inorganic salts which interfere with the partition chromatography.

This column, $3 \times 0.5 \text{ cm}$, was packed from a suspension of talc in distilled water, under these conditions the porphyrin is adsorbed on the top of the column with the other pigments beneath it. The column was then washed with distilled water until the eluate was free from Cl^- . Any remaining non fluorescent pigments were then removed by washing the column with increasing concentrations of aqueous NH_3 , beginning with 0.01N, the porphyrin being finally eluted with 10N NH_3 in which it descends as a narrow band and can be collected in approximately 0.1 ml of solution which was applied to the paper chromatogram.

The specimen of Fischer's conchoporphyrin ester (m.p. 270–273°, Fischer & Jordan, 1930) was prepared for chromatography by hydrolysing it in approximately 7N HCl for 24 hr at room temperature. The HCl was removed in a vacuum desiccator over KOH and the porphyrin residue dissolved in 0.1 ml of aqueous 10N NH_3 .

Partition chromatography

The partition chromatograms were run on strips of Whatman no. 1 filter paper ($6 \times 40 \text{ cm}$) at a temperature of 21°, using a mixture of the 2,4- and 2,5-dimethylpyridines

('lutidine') as solvent, and in an atmosphere saturated with the solvent and water vapour.

Porphyrins are separated under these conditions into spots clearly visible by their pink fluorescence in ultraviolet light. It has been shown that the R_F values of the spots bear an inverse linear relationship to the number of carboxyl functions in the porphyrin molecule which they represent (Nicholas & Rimington, 1949).

The R_F values at 21° for representative porphyrins are recorded in Table 1.

Table 1 R_F values of porphyrins in 'lutidine' at 21°

Porphyrin	R_F values	No of carboxyl functions
*Uroporphyrin	0.07 (0.12)	8
Coproporphyrin	0.5	4
Protoporphyrin (and other 2 carboxyl porphyrins)	0.75	2
Phylloerythrin	0.87	1
All esters	0.98	0

* Uroporphyrin characteristically gives two spots

In order to avoid misinterpretation through slight changes in R_F due to variation in conditions, known markers were run on the strip in every case alongside the material under investigation.

RESULTS

The results which we have obtained with eight different types of shell and with Fischer's conchoporphyrin (Fischer & Jordan, 1930), stated to be derived from *Pteria radiata*, are recorded in Table 2.

Table 2 R_F values and identification of porphyrins present in materials examined

(The R_F value in brackets represents the additional uroporphyrin spot.)

Shell	R_F values of spots obtained	Porphyrin present
Fischer's 'concho porphyrin' (<i>Pteria radiata</i>)	0.06 (0.11) 0.49	Uroporphyrin and coproporphyrin
<i>Pteria radiata</i> from S. Carolina	0.07 (0.12)	Uroporphyrin
<i>Pinctada vulgaris</i> (<i>Pteria vulgaris</i>)	0.07 (0.12) 0.49	Uroporphyrin and coproporphyrin
<i>Trinia europæa</i>	0.07 (0.12)	Uroporphyrin
<i>Bulla</i> sp.	0.08 (0.14)	Uroporphyrin
<i>Placuna sella</i>	0.05 (0.1)	Uroporphyrin
<i>Umbonium australe</i>	0.08 (0.13)	Uroporphyrin
<i>Malleus vulgaris</i>	0.06 (0.11)	Uroporphyrin
<i>Gibbula cineraria</i>	0.07 (0.11)	Uroporphyrin

DISCUSSION

The results confirm earlier evidence that the porphyrin present in shells is uroporphyrin, accompanied in some cases by smaller quantities of coproporphyrin. No evidence has been found for the existence of a penta- or hepta-carboxylic porphyrin.

The fact that the uroporphyrin does not behave as an entity on the chromatogram would point to the possibility of a seven carboxyl porphyrin (R_f 0.12) being present. This has been excluded on the grounds first, that other samples of uroporphyrin of non-molluscan origin, considered pure by all other standards, behave identically, and secondly that the 'spot' of R_f 0.07, which represents the majority of the porphyrin, can be shown to decompose again into two spots (R_f 0.07 and 0.12) by rotating the chromatogram through 90° and re running in the new direction with the same solvent.

There exists an element of doubt surrounding Fischer's identification of his shell material (Fischer & Jordan, 1930), his published papers deal specifically with two species of *Pteria*, viz *Pteria radiata*, described as a Venezuelan species from which Fischer & Jordan (1930) obtained conchoporphyrin, and *Pteria vulgaris*, now called *Pinctada vulgaris*, which is widely distributed in the Indian Ocean and used in the button and fancy goods trade under the name of Persian lingah shell.

The specimens of *Pteria radiata* Lk * which we have examined were from South Carolina and their identity was kindly established by Dr John Burch of Los Angeles who supplied them. We have also examined carefully identified *Pinctada* (*Pteria*) *vulgaris* from Bahrein on the Persian Gulf. These were obtained from the dealer who supplied Fischer with his specimens of *P. vulgaris*.

In view of the uncertainty, both chemical and zoological, surrounding Fischer's material, it would be difficult to express a definite opinion as to the existence of his 'conchoporphyrin', but the fact that we found his ester material, m.p. $270-273^\circ$, to be a mixture of uroporphyrin and coproporphyrin, casts

* A photograph of the shell may be obtained from the authors.

doubts upon his claims. Certainly no pentacarboxylic porphyrin is present in any of the species of shell we have examined.

The uniformity which we find between the porphyrins of different groups of shells might have been predicted if it is assumed that they are end products of a closely similar or identical metabolic cycle. It is of interest that no porphyrins of the chlorophyll series have been encountered.

Although we have not been able to confirm the existence of conchoporphyrin, it may be mentioned that the technique of partition chromatography has revealed in other materials the existence of porphyrins presumably containing five and seven carboxyl groups respectively (Rimington, personal communication).

SUMMARY

1. Extracts of porphyrin containing molluscan shells have been examined by adsorption and paper partition chromatography.

2. The presence of uroporphyrin and, in one species, of coproporphyrin has been shown.

3. No evidence has been found for the existence in these shells of the pentacarboxylic porphyrin described by Fischer & Jordan (1930) under the name of 'conchoporphyrin'.

We wish to express our thanks to Prof C. Rimington for his help and encouragement and for access to unpublished data, to Prof J. Waldenström of Uppsala for a specimen of Fischer's conchoporphyrin, to Dr E. M. Jope, Medical Research Council Spectrographic Unit, London Hospital, for assistance with spectrographic measurements and to Miss A. Benson, for technical assistance. The work was financed by the Yarrow Research Fund (London Hospital) and by a grant from the Medical Research Council. The substance of this, and the foregoing three papers on molluscan pigments in this series, has been approved as part of a Thesis for the degree of Ph.D. in the University of London.

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Distribution and Nutritive Value of the Nitrogenous Substances in the Potato

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(Received 26 January 1949)

In a former communication, Chick & Cutting (1943) reported a series of experiments in which the nutritive value of the proteins and other nitrogenous substances of the potato was compared with that of casein and of the proteins in wheat. The criterion adopted was the ability to support growth in weanling rats fed on diets adequate in respect to other essential nutrients, but containing a sub-optimal amount of nitrogen derived solely from the food under test. Under these conditions, in which the nitrogen is the factor limiting growth, the relative rates of growth provide a basis for the comparison of the nutritive values of the mixtures of proteins (Chick, 1947, Slack, 1948b).

By the above method the nutritive value of the nitrogen of the potato was found to be somewhat superior to that of wheat nitrogen, although the digestibility was lower. This result was the more remarkable, since less than half of the nitrogen of the potatoes studied was present as protein, and about 60% consisted of amides, nitrogenous bases and free amino acids. The non-protein nitrogen contained in the expressed juice, after removal of the protein material by heat coagulation at pH 4, was unable to support growth. Nevertheless, the mixture of protein and non-protein nitrogen in the whole potato had a nutritive value at least equal to that found for the separated protein material.

The fact that the nitrogen of the potato, weight for weight, was as efficient as that of wheat for maintenance of nitrogenous equilibrium in adult man or animals, has been abundantly demonstrated (Rubner, 1879, Hindhede, 1913, Abderhalden, Ewald, Fodor & Rose, 1915, Rose & Cooper, 1917, Rubner & Thomas, 1918, Kon, 1928, Kon & Klein 1928). The reason why this should be so, seeing that 50% or more of the potato nitrogen is non-protein in nature, puzzled Rubner 70 years ago. The puzzle remains unsolved, in spite of the advances in knowledge of proteins and nutrition which have been achieved since the period when Rubner was confronted with it (see p. 219).

Proteins of the potato

A protein having the properties of a globulin, to which they gave the name *tuberin*, was found by Osborne & Campbell (1895-1896) in the expressed juice of the potato and was

extracted from the residual pulp with NaCl solution. Since the protein materials from both sources had similar elementary compositions and precipitation limits with $(\text{NH}_4)_2\text{SO}_4$, it was concluded that they were the same. They could be purified by dialysis and heat coagulation took place irregularly between 56 and 76°. The purified material contained 16.24% N. Neuberger & Sanger (1942), in an investigation of ten varieties of potatoes, the total N content of which varied from 1.16 to 1.95% (on dry weight), found the proportion of protein N to vary from 28 to 51% of the total soluble N. Of the non-protein N, 34% (range 19.4-42.1%) consisted of amide N, glutamine and asparagine, 10% of $\alpha\text{-NH}_2$ N (range 6.4-12.8%) and 23% of basic N (range 20-28%). A residue of the total N in the potato amounting to about 10% could not be extracted by methods which included extractions with water, salt solution, weak alkali, ethanol, ethanolic alkali and urea. This N was assumed to be protein in nature and was present mainly in the skin and cortex of the tuber. Groot, Janssen, Kentie Oosterhuis & Trap (1947) found Osborne & Campbell's (1895, 1896) *tuberin* to be a mixture of two proteins which were precipitated from dilute press juice at different pH values. For the globulin precipitated at the higher pH they retained the name *tuberin*, for the other they suggested the name '*tuberinin*'. Separation could also be made by electrophoresis of the press juice and by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$. The relative amounts of *tuberin* and *tuberinin* present were about 71/29. Jørgensen (1946) concluded that, owing to the labile nature of the proteins in potato juice, several different proteins could be separated according to the methods used.

The work reported in the present paper forms a continuation of that already published (Chick & Cutting, 1943), by a further study of the complementary nutritive action of the non-protein nitrogen of the potato sap for the protein *tuberin*. In the search for an explanation of this complementary effect, an analysis of these fractions for their amino acid content was essential. Another aim was to recombine the different isolated nitrogenous fractions, including any insoluble protein that might be present, in an attempt to reconstitute the nitrogenous mixture as it exists in the intact tuber, an attempt which has proved only partially successful.

The difficulty experienced in maintaining fresh potatoes in a stable condition for a continued study led to a research on the changes in distribution and in nutritive value of the nitrogen, which takes place

under different conditions of storage and to the use, where possible, of dried material for the tests. With this work was combined an investigation of the relative nutritive value of the nitrogenous materials situated in different portions of the tuber.

EXPERIMENTAL

Materials and methods

Animal tests The general technique employed was similar to that described previously, the criterion of nutritive value being the growth promoting efficiency of the nitrogenous substances for weanling rats (Chick, 1942, Chick & Cutting, 1943).

Potatoes The potatoes were of the 'King Edward' variety grown in the Fenlands. In many experiments in which nitrogenous fractions were investigated, the mixture of nitrogenous substances contained in the intact potato was used as a standard of comparison. When the potatoes were steamed, skinned, mashed and dried at a temperature not exceeding 40° in a current of air, the nutritive value of the N of the dried material was identical with that of freshly steamed, skinned potatoes (Chick & Cutting, 1943), and if the dried material was stored in closed tins in a cool cellar, no serious subsequent deterioration was observed.

Diets made with dried whole potato were kept in the dry state, and, immediately before feeding, water was added in about the proportion present in the original fresh potato. Diets containing as source of N the separated nitrogenous fractions, or mixtures of these which were uncooked, were mixed with an appropriate amount of water and cooked in a steamer for about 1 hr. The pudding like material obtained was found convenient to feed.

Potato protein The total heat-coagulable globulin of potato sap, the tuberin of Osborne & Campbell (1896), was separated from the juice expressed from raw, washed, minced potatoes by a method based on that of Kon (1928). After adjusting the pH of the press juice to 4.5 it was heated to 80° on a water bath with continuous stirring for about 1 hr. The precipitate was allowed to settle, was centrifuged, washed with water, ethanol, acetone and ether and finally dried on a water bath. In the best preparations it was a light grey powder, but, if allowed to take up any moisture during the removal of the organic solvents, it rapidly became discoloured and coarser in texture. Material prepared in this way contained about 5–10% moisture and 14–15% N on the dry weight, the ash content averaged about 4%.

The protein material, as prepared by the above method and referred to as tuberin in this paper, contains the total heat-coagulable proteins in the potato sap and includes the two globulins separated by Groot *et al.* (1947) and others, as well as by ourselves (see below). It does not, however, include the 10% of insoluble protein presumably combined with the residual pulp, according to the observation of Neuberger & Sanger (1942).

The N of this tuberin had an average digestibility coefficient of about 63% (range 56–70%). Even when great care was taken in its preparation the highest digestibility recorded was still considerably lower than the average coefficient (76%) for the N of the whole potato.

Crude tuberin In order to minimize any damage to the digestibility of the tuberin, use was sometimes made of crude preparations of protein obtained by washing the centrifuged

protein sludge with water only, and either incorporating this material directly into the diet or drying it at low temperature in a current of air. Such preparations contained about 14% N on the dry weight and had a digestibility coefficient of 77%, i.e. about equal to that of the whole potato.

Protein free juice The filtrate of the juice after removal of the precipitated tuberin contained 0.3 g N/100 ml. It was mixed with the aqueous washings of the tuberin, concentrated *in vacuo* at 40°, adjusted to pH 3 and stored in the refrigerator until required. When fed alone or as the major source of N in the diet, it caused severe diarrhoea, but this could usually be prevented by adding to the diet about 1.5% of shredded filter paper to provide roughage. The N contained in this juice was nutritionally adequate for maintenance, but proved barely capable of supporting growth even when fed at a level of 3% on the dry weight of the diet.

Potato pulp After juice had been expressed from the raw, minced potatoes, the pulp was washed by suspension in water and pressed as dry as possible, at which stage it contained about 50% water. It was then dried in a current of warm air and powdered in a hammer mill. The weight ratio of the pulp (dry weight) to the expressed juice was about 20/80, the pulp contained about one fifth of the total N of the potato.

Chemical studies

Distribution of the nitrogen in the potato The potatoes used contained from about 1.7 to 2.0% N on the dry weight, the amount present as protein varying from about 40 to 53% of the total. These values varied, however, with the age of the potatoes and conditions of storage, one sample contained 47% protein N when fresh and 43% after 10 months in the chamber at 5°.

In the sample used about one fifth of the non protein N in the juice was present as free amino acids, two fifths as amides and two fifths as nitrogenous bases (see also Neuberger & Sanger, 1942, Chick & Cutting, 1943). The press juice from the washed, minced, raw potatoes usually contained about 0.45 g N/100 ml, about one-third of this was protein and about two-thirds non protein N.

We were able to detect at least two proteins in potato press juice. When the diluted press juice, from which the starch had been largely removed by filtration through thick chain cloth and subsequent sedimentation, was adjusted to pH 4 and allowed to stand, a protein which may be called 'α globulin' precipitated out, the separation being facilitated by gentle warming in an incubator at 30°. A second protein, 'β globulin' was precipitated when the supernatant solution was boiled. In a sample of freshly dug potatoes the proportion of α to β globulin was about 30/70, in a sample of old potatoes after storage for 1 year, these values were reversed and were about 65/35. One specimen of β globulin which was analysed contained 15.6% N.

The amino acid composition of tuberin The total heat-coagulable protein in the press juice, as prepared by the method of Kon (1928), was analysed by one of the authors (E B S) for its content of the undermentioned essential amino acids. The methods used were those of Gale (1946) for lysine, Tristram (1946) for valine, leucine, tyrosine and phenylalanine, Macpherson (1946) for arginine and histidine, Lugg (1938) for cystine and methionine, Hess & Sullivan (1945) for tryptophan and Rees (1946) for threonine. The protein free filtrate, from which the tuberin had been removed, was concentrated *in vacuo*, the amino acid fraction

separated by the procedure of Vickery (1925) and analysed for essential amino acids by the above mentioned methods

The results, which were published recently (Slack, 1948a), are summarized for reference in Table 1. It will be seen that the tubern is richer in the ten essential amino-acids determined than is the amino acid fraction of the N P N. If it is assumed that all the protein in the potato has the same composition as tubern, the amino acid composition of the 'crude' protein (N x 6) of the whole potato can be calculated. The values obtained (column 3, Table 1) show the degree to which the content of essential amino acids in the 'crude' protein of the whole potato is lower than in tubern. The significance of this result is dealt with below when the nutritive value of the different nitrogenous fractions is discussed.

Table 1 *Amino acid content of potato sap*

	Amounts of essential amino acids expressed as g/16 g N in		
	(1) Tubern fraction	(2) N P N fraction	(3) Mixture of 53 parts (1) with 47 parts (2)*
Phenylalanine	6.6	4.1	5.4
Leucines	17.5	4.3	11.3
Valine	6.1	3.3	4.8
Tryptophan	1.6	—	0.8
Threonine	5.9	1.1	3.7
Arginine	6.0	2.6	4.4
Histidine	2.2	1.1	1.7
Lysine	7.7	1.9	5.0
Cystine	2.1	1.2	1.6
Methionine	2.3	0.8	1.6

* The proportions in which the protein and N P N were present in the sample of potatoes used.

The figures given by Groot (1945) are not strictly comparable with those obtained in the present work. Groot's values refer to 100 g tubern of 13.9% N content whereas those in the present work are calculated per 16 g N (see

Table 1). Nevertheless, except for lysine content, for which Groot's figure is much lower, the divergencies are not too great considering the difficulties of this type of analysis. It must also be remembered that the values given in this paper for the amino acid composition of the tubern and the N P N are applicable only to the particular sample of potato studied for, even though tubern itself should be of constant composition, the variations which are known to occur in the relative proportions of protein and N P N are sufficient to cause great variation in the amino acid content of the whole potato.

Feeding experiments

(a) *Effect of storage upon the distribution and nutritive value of the nitrogenous constituents of the potato (Exp 1a, b, c)*

Samples of three batches of King Edward potatoes (6, 8 and 10) were dried, after steaming and removal of the skin, within 1 month of lifting. Other samples of the same batches were similarly dried after storage for several months, those of batch 6 after storage for 10 months in a chamber maintained at 5°, those of batch 8 for 5 months in a clamp in the open followed by a further 5 months in a chamber at 5°, those of batch 10 for 5 months in a clamp. Analysis of the potatoes of batches 6 and 8 after storage for 10 months showed a slight increase in the N content on the dry weight, indicating loss of non nitrogenous material during storage, presumably due to respiration. In the case of the potatoes of batch 10, which had been stored for 5 months only, this change was not observed (Table 2).

The above six samples of dried potatoes were incorporated as source of N in six diets, nos 72 and 73, 74 and 75 and 122 and 123, respectively. Potato starch was included where necessary to equalize the N contents of the diets, further details of the composition of the diets are given in Table 2. The animal tests with the first four of the above diets were made simultaneously (in Exp 1a, b), those with diets 122 and 123 some time later (in Exp 1c). In each case the diets were fed to comparable groups of 6-8 litter mate weanling rats for a period of 7 weeks, the food intake and the economy of utilization of the N eaten was measured during 1 week in

Table 2 *Composition of materials and diets used in Exp 1*

(The proportions of constituents in the diets are given in parts by weight)

Materials	Batch no	State of potatoes	Moisture (%)	N (% of dry wt)	Protein N (% of total N)	Exp 1a		Exp 1b		Exp 1c	
						Diet 72	Diet 73	Diet 74	Diet 75	Diet 122	Diet 123
Dried King Edward potato	8	Newly lifted	12.6	1.88	42.8	100	—	—	—	—	—
	8	Stored 5 months in clamp + 5 months at 5°	10.9	2.04	37.3	—	91	—	—	—	—
	6	Newly lifted	12.2	1.82	47.2	—	—	100	—	—	—
	6	Stored 10 months at 5°	12.1	1.86	42.9	—	—	—	98	—	—
	10	Newly lifted	10.7	1.96	—	—	—	—	—	93	—
	10	Stored 5 months in clamp	10.7	1.96	—	—	—	—	—	—	93
Potato starch			10 (approx)	0.01	—	—	7	—	2	—	—
Hardened arachis oil						4	4	4	4	4	4
Salt mixture*						2	2	2	2	2	2
Cod liver oil						1	1	1	1	1	1
Average N content of diet (% of dry wt)†						1.73	1.70	1.66	1.65	1.78	1.80

* McCollum, Simmonds & Pitz (1917)

† Over whole 7 week period of the tests

Exp 1a, b and during 2 weeks in Exp 1c Average growth curves of the rats in all three experiments are presented

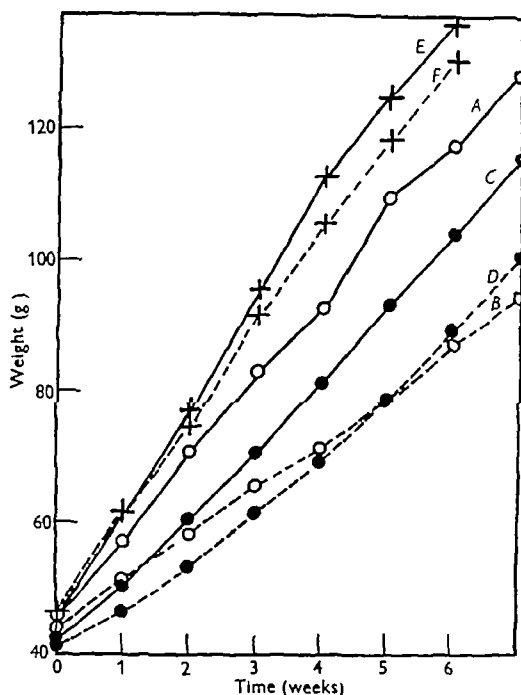


Fig 1, Exp 1a-c Average weight curves of rats maintained on diets containing nitrogen from potatoes dried as follows

Curve	Potato sample	Dried	Diet	N in diet (% of dry wt)
A	KE 8	Freshly dug	72	1.73
B	KE 8	Stored 10 months	73	1.70
C	KE 6	Freshly dug	74	1.66
D	KE 6	Stored 10 months	75	1.65
E	KE 10	Freshly dug	122	1.78
F	KE 10	Stored 5 months	123	1.80

together in Fig 1. In each case the rats, having diets containing potatoes dried immediately after lifting, had a higher

rate of weight increase than those receiving potatoes dried after storage, although there was variation in the extent of the difference. The greatest deterioration was observed in the potatoes of batch 8 after storage for 5 months in a clamp, followed by a further 5 months in a chamber at 5°, there was a smaller but considerable decline in the nutritive value of batch 6 stored for 10 months at 5°. The average weekly weight increase over a 7 week period of the rats receiving fresh and stored samples of these two batches were, respectively, 18.4 and 13.5 g (batch 8) and 16.5 and 14.4 g (batch 6). With the potatoes of batch 10, which had been clamped for 5 months only, the deterioration was less marked. Nevertheless, the economy with which the N of this batch was utilized was considerably reduced by the storage, as indicated in Table 3, which gives the average performance/rat/week on each of the six diets. The same was true of the potatoes of batch 8, but was not observed with those of batch 6 during the actual week of measurement of the utilization.

These results indicate that the changes taking place in the nitrogenous constituents of the potato during storage, lead to a deterioration in their nutritive value.

(b) Nutritive value of the nitrogen of the whole potato with and without the peel, compared with that of various separated nitrogenous fractions fed alone or in various combinations

Exp 2. Comparison of the nutritive value of the nitrogen of whole potato, potato minus skin and potato minus skin and outer cortex. The skin and outer layers of the potato are richer in total N than the inner layers, and also contain a higher proportion of insoluble N than the rest of the tuber (Neuberger & Sanger, 1942). In one sample of potatoes, examined soon after lifting, the skin accounted for 7.6% of the dry weight of the tuber and for about 10% of the total N. Thus it seemed desirable to determine whether the N contained in the skin and outer layers was of greater nutritive value than that in the inner parts, or had any supplementary value for this. Comparison was therefore made of the growth supporting value of diets in which the N was supplied by (a) steamed whole potato, (b) the same potatoes steamed after removal of the skin, and (c) potatoes peeled before steaming to remove the skin and a layer about 2 mm deep of the cortex. The potatoes used for the test (batch 6) were

Table 3. Food intake and economy of utilization of nitrogen on diets nos 72-75 and 122, 123

(Average figures/rat/week.)

Exp no	Diet no	Batch no	State of potatoes	No of rats	N in diet* (%)	Dry food eaten (g)	N eaten (g)	Average wt* increase (g)	Coefficient† of (apparent) digestibility of N	Wt increase	
										(g/g N eaten)	(g/100 g dry food eaten)
1a	72	8	Newly lifted	8	1.72	78.0	1.34	17.0	—	12.7	21.8
	73	8	Stored	8	1.67	58.7	0.98	7.7	—	7.9	13.2
1b	74	6	Newly lifted	8	1.64	71.7	1.17	9.6	—	8.2	13.4
	75	6	Stored	8	1.67	71.4	1.20	10.5	—	8.8	14.7
1c	122	10	Newly lifted	6	1.78	85.3	1.51	18.7	76.9	12.4	21.9
	123	10	Stored	6	1.80	81.6	1.47	14.0	78.9	9.5	17.2

* Average values during periods in which food intake was measured. Exp 1a fifth week, Exp 1b, sixth week, Exp 1c, third and fourth weeks.

† $\frac{N \text{ eaten (g)} - N \text{ in faeces (g)}}{N \text{ eaten (g)}} \times 100$

steamed and dried within 2 months of lifting and had been stored in a clamp in the open

cortex in diet 50 resulted in a lowering of the digestibility of the N, the coefficient being 74.2% as compared with 77.8% for diet 51 and 78.6% for diet 52

Table 4 *Composition of materials and diets used in Exp 2*

Material	Dry matter (%)	N (% of dry wt)	Diet 50 (g)	Diet 51 (g)	Diet 52 (g)
Steamed whole potato (batch 6)	22.2	1.72	500	—	—
Steamed potato minus skin	22.0	1.67	—	500	—
Steamed potato minus skin and outer cortex	21.2	1.63	—	—	500
Maize starch	90	0.03	8	5	—
Hardened arachis oil			4	4	4
Salt mixture*			2	2	2
Cod liver oil			1	1	1
Average N content of diet (% of dry wt)†			1.54	1.56	1.58

* McCollum *et al* (1917)

† Over whole period of test, 7.5 weeks

Details of the three diets used (50, 51 and 52) are given in Table 4. They were fed to three groups of litter mate rats for 7.5 weeks and measurements of food intake and of digestibility of the N were made during four successive weekly periods.

From Fig. 2 it is seen that the best growth rate was obtained on diet 52 which contained the inner portions only of the potato, the average weekly weight increase over the whole period of the test being 13.9 g. The corresponding figures for the diets containing the skinned and unskinned potatoes were 12.7 and 11.8 g respectively.

In this, as in all cases involving the nitrogenous material in the potato, it is not possible to speak of the 'protein efficiency ratio', or the weight increase/g protein ingested, seeing that so great a proportion of the N is of a non protein nature. The ratio, weight increase (g)/N ingested (g) may, however, be substituted. The values of this ratio corresponding to diets 50, 51 and 52 for the 4-week period in which the food intake was measured were respectively 9.5, 10.2 and 11.4 (Table 5). The inclusion of the N of the potato skin and outer

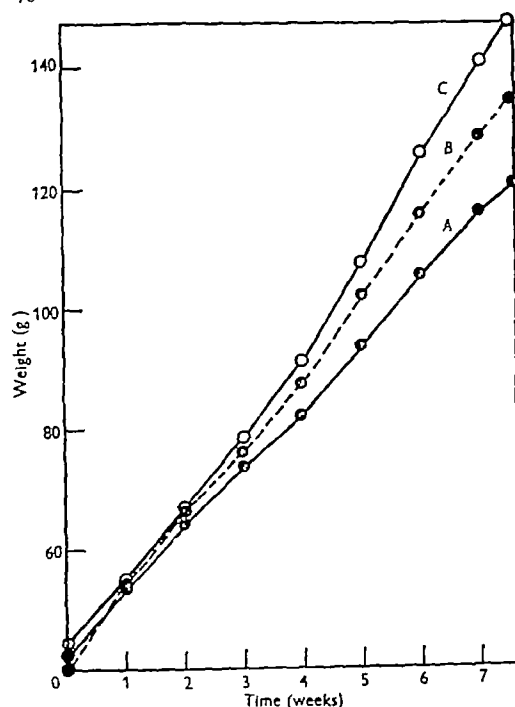


Fig. 2, Exp. 2 Average weight curves of eight rats maintained from weaning on diets containing 1.54–1.58% N (on dry weight) derived from potatoes (sample KE 6). A, potatoes dried after steaming with skin retained (diet 50), B, potatoes dried after steaming, skin removed (diet 51), C, peel to a depth of about 2 mm removed from the potatoes before steaming (diet 52).

The slightly lower digestibility of the N in diet 51, which contained the potato skin, was not considered responsible for the inferior growth supporting capacity of this diet. This conclusion was confirmed by the results of a further

Table 5 *Exp. 2 Food intake and economy of utilization of nitrogen on diets nos 50–52*

		(Average figures/rat/week.)									
Diet no	Source of N	No of rats	N in diet* (%)	Dry food eaten (g)	N eaten (g)	Faeces passed (dry wt, g)	N in faeces (g)	N digested (g)	Coefficient† of (apparent) digestibility of N	Average wt increase (g)	Wt increase (g/g N eaten) (g/g N digested)
50	Whole potato (batch 6)	8	1.53	71.1	1.09	5.42	0.281	0.809	74.2	10.4	9.5 12.9
51	Whole potato (batch 6) minus skin	8	1.55	74.5	1.15	4.82	0.255	0.896	77.8	11.8	10.2 13.2
52	Whole potato (batch 6) minus skin and outer cortex	8	1.58	80.3	1.27	5.40	0.271	0.997	78.6	14.4	11.4 14.3

* Average during 4-week period in which food intake was measured

† $\frac{N \text{ eaten (g)} - N \text{ in faeces (g)}}{N \text{ eaten (g)}} \times 100$

Table 6 *Composition of materials and diets used in Exps 4, 5 and 6*

(The proportions of constituents in diets are given in parts by weight. Dried potato diets (35.4, 73.4, 107, 107.4) before feeding were mixed with water in amount about equal to that present in the fresh potato, other diets were mixed with water and steamed for 1-1.5 hr.)

Maternal	Exp 4			Exp 5			Exp 6		
	Moisture (% approx.)	N (% of dry wt.)	Diet	Moisture (%)	N (% of dry wt.)	Diet	Moisture (%)	N (% of dry wt.)	Diet
Potato (dried)	11	1.93	35.4 93	12.0	1.97	73.4 91	+skin 10.7 -skin 11.4 crude 6.8	1.93 2.02 11.1	107 104 — 93 — 3.7
Potato protein (tuberin)		11.85 (air-dry)	—	6.2	14.0	—	13 10	—	— 8.8
Whole press juice (concentrated)	80	1.32-1.97* (g/100 ml.)	—	—	—	—	—	—	—
Protein free juice (concentrated)		0.91	—	15.6	0.48	—	(100 ml.) 80.8 crude 13.9	1.95 0.81	— 39 — 95.5 — 40.5
Potato starch			—	—	0.01	7 91	—	—	—
Milze starch	10	0.03	—	—	—	—	—	—	—
Hardened arachis oil			4	—	—	4 4 4	—	—	—
Salt mixture†			2	15-23	2	2 2 2	—	—	—
Cod liver oil			1	1	1	1 1 1	—	—	—
Filter paper			—	12-14	—	—	—	—	—
Average N content of diet §			1.70 1.80 1.81		1.65 1.69 1.67		1.80 1.77 1.74		1.79

* According to degree of concentration

† McCollum *et al.* (1917)

‡ According to N content of concentrated juice

§ Over whole period of test Exp 4, 6 weeks, Exp 5, 6 weeks, Exp 6, 7 weeks

Table 7 *Exps 4, 5 and 6 Food intake and economy of utilization for growth of the nitrogenous substances present in the whole potato and their different fractions*

Exp no	Diet no	Source of nitrogen	(Average values/rat/week)					Coefficient of (apparent) digestibility of N†	Average wt increase* (g)	Wt increase	
			No of rats	N in diet* (%)	Dry food eaten (g)	N eaten (g)	Faeces passed (dry wt g)	N in faeces (g)	N digested (g)	(g/g N eaten)	(g/100 g food eaten)
4	35.4	Dried potato (batch 1, without skin)	5	1.70	64.3	1.10	5.60	0.269	0.83	75.4	11.3 19.3
	36.8	Press juice	5	1.80	56.1	1.02	3.91	0.173	0.85	83.1	9.5 17.3
	39	Tuberin	4	1.81	54.5	0.99	4.07	0.355	0.63	64.0	11.0 20.0
5	73.4	Dried potato (batch 8, without skin)	2	1.65	89.1	1.47	5.72	0.306	1.16	79.2	9.5 15.7
	76	Tuberin	3	1.67	69.3	1.55	5.33	0.503	0.65	56.4	8.3 13.8
6	107	3/4 from tuber in 1/4 from washed pulp	3	1.63	59.3	0.98	6.58	0.515	0.47	47.5	6.8 11.2
	107.4	Dried potato (batch 10, with skin)	5	1.82	57.6	1.05	5.10	0.249	0.80	76.3	7.2 13.2
108	107.4	Dried potato (batch 10, without skin)	5	1.78	62.6	1.11	1.02	0.250	0.85	76.8	10.1 17.9
	108	1/5 from crude tuber in 2/5 from unwashed pulp 2/5 from protein free juice	5	1.74	55.3	0.96	5.23	0.297	0.67	69.2	5.8 10.5
109	109	1/2 from crude tuber in 1/2 from protein free juice	5	1.76	56.7	1.01	3.74	0.297	0.71	70.6	7.9 13.9

* Average during period in which food intake was measured Exp 4, 6 weeks, Exp 5, 3 weeks, Exp 6, 2 weeks

† $\frac{N \text{ eaten (g)} - N \text{ in faeces (g)}}{N \text{ eaten (g)}} \times 100$

experiment (Exp 6, Table 7) made for a different purpose, in which the control tests with whole potatoes included trials of those dried with and without the skin (diets 107 and 107A, Table 6). The performance of the rats receiving the former was markedly inferior to that of those receiving the dried, skinned potatoes as regards weight increase and utilization of N and of total calories. The average weekly weight increases over a 7 week period were respectively, 8.2 and 10.8 g, the ratio, weight increase (g)/N ingested (g), over a 2-week period in which food intake was measured, was respectively, 7.2 and 10.1. The digestibility coefficients of the nitrogen in the two diets were 76.3 and 76.8% respectively.

Exp 3 The nutritive value of the nitrogen in the protein-free juice fed alone, or mixed with tuberin, compared with that of the nitrogen of the whole potato. A diet was prepared in which the nitrogen was provided by the protein free juice of the potato,

in the whole press juice (ratio 1:2), the average weekly weight increase during the following 2 weeks was 9.1 g (Fig 3, Curve B₁). When, during the next 3 weeks, the proportion of protein to N:P.N was adjusted to that in the sample of whole potato used (ratio 44/56), the average weekly weight increase was 6.8 g (Fig 3, Curve B₂). These results show the supplementary relation of the N:P.N for that of tuberin, seeing that on a diet containing the same amount of N derived from the protein alone the average weekly weight increase was 9.6-9.7 g (Table 7, Exps 4 and 5). The remaining three rats in Exp 3 were given a similarly composed diet containing 1.7% nitrogen provided from the whole potato. During the following 5 weeks steady growth occurred with an average weekly weight increase of 1.9 g, showing that in the case of the whole tuber the mixture of protein and N:P.N nitrogen was even more efficient.

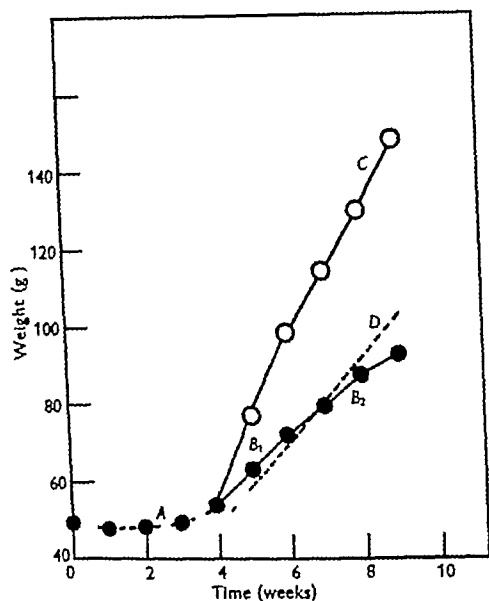


Fig 3, Exp 3 Average weight curves of weanling rats maintained on diets containing N derived from A, protein free press juice (1.9-3.0% N on dry wt), B₁, tuberin N and non protein N in proportion of 1/2, B₂, in proportion of 44/56 (1.7-1.8% N on dry wt), C, whole dried potato (minus skin, 1.7% N on dry wt), D, tuberin (1.7% N on dry wt) from Exp 5

it consisted of 95 parts of concentrated juice containing 1.73% N and 20% dry matter, together with potato starch 71, hardened arachis oil 4, salt mixture 2, and 1 part each of cod liver oil and shredded filter paper, the average N content on the dry weight was 1.9%. It was fed to a group of six young rats. After 2 weeks on this regime, during which there was no appreciable gain in weight, the proportion of juice in the diet was increased to raise the N content to 2.3% on the dry weight without effect and in the following week to 3.0%, at which level the animals gained an average weight of 4.3 g. When, however, at the end of the fourth week, three of the animals were given a similarly composed diet containing 1.7-1.8% N on the dry weight, provided by a combination of tuberin with protein free sap, to provide N from these in the proportion in which they were present

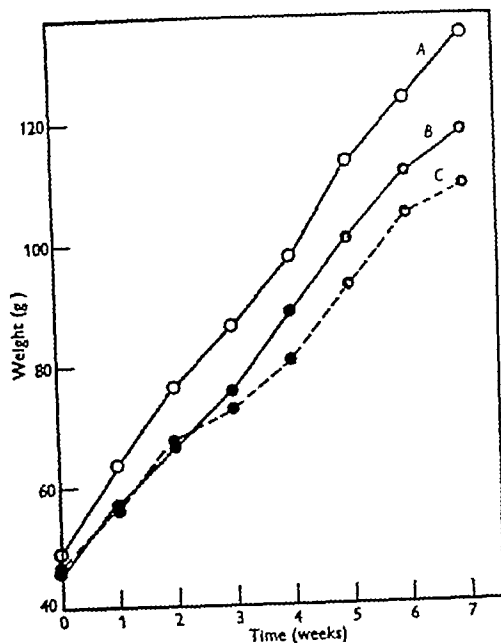


Fig 4, Exp 4 Average weight curves of four to five rats maintained from weaning on diets containing 1.7-1.8% N (on dry wt) derived from A, whole dried potato (diet 35A), B, tuberin separated from the press juice (diet 39), C, whole press juice (diet 36B)

Exp 4 The nutritive value of the nitrogen (protein and non protein) in the expressed whole juice, and of that of the protein (tuberin) separated from the juice, compared with that of the nitrogen of the whole potato. Diets 36B, 39 and 35A, made to contain about 1.7-1.8% N (on the dry weight), derived from the sources given in the above heading, were fed to three comparable groups of 4-5 litter mate rats from weaning (see Table 6). The results of this experiment are summarized in Table 7, and the average growth curves of the rats in the three groups are given in Fig 4.

The performance of the rats receiving the whole dried potato as source of N was again better than that of those receiving the mixture of protein and N:P.N as these are present in the whole press juice, the average weekly weight increases over a period of 6 weeks being 12.4 and 9.7 g

respectively. The digestibility of the N in the latter, which contained simpler nitrogenous material, was naturally higher (coefficient 83%) than that of the former (coefficient 75.5%). The N in the separated tuberin had also a lower digestibility than that of the whole potato, and the weekly weight increase (110 g) was also lower. The ratios, weight increase (g)/N consumed (g), were similar for the whole potato (11.3) and tuberin (11.0) and somewhat lower (9.5) for the press juice.

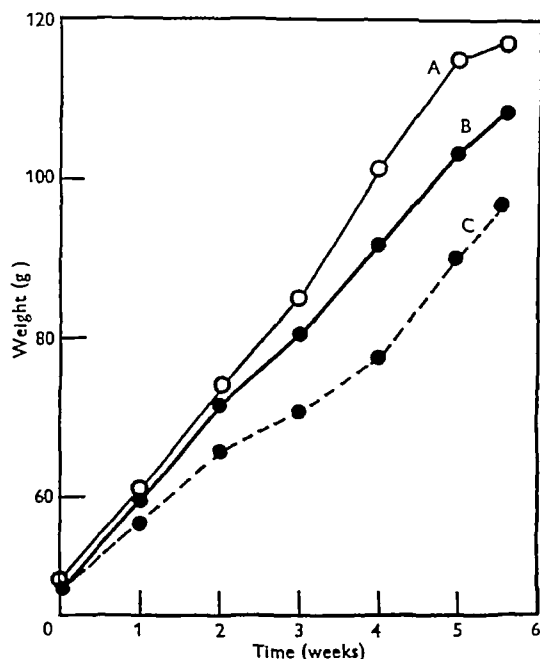


Fig 5, Exp 5 Average weight curves of two or three rats maintained on diets containing 1.65-1.70% N (on dry wt) derived from potato, sample KE8. A, whole potato dried without skin (diet 73A), B, separated protein (tuberin, diet 76), C, $\frac{2}{3}$ from tuberin, $\frac{1}{3}$ from washed pulp (diet 77).

The results with diets 36B and 39 demonstrate again the supplementary effect of the N in the protein free fraction of the sap for that of the tuberin fraction.

Exps 5 and 6 These were in essentials repetitions of Exp 4, but more care was taken in the separations of the different fractions. In Exp 5 all the fractions were more thoroughly washed, in Exp 6 all processes were speeded up and washing of the fractions omitted. The N in the three diets used in Exp 5, which contained 1.65-1.7% N on dry weight, was provided by whole potato dried after removal of the skin (diet 73A), or by washed dried tuberin (diet 76), or by a mixture of tuberin and the washed pulp left after pressing the juice from the raw minced potato (diet 77, in which the pulp provided about one quarter of the N, a proportion similar to that provided by the pulp in the whole potato). All these materials were derived from the same batch of King Edward potatoes, but there was an interval of 3-6 months between the preparation of the whole dried potatoes and that of the tuberin and pulp, during which the potatoes were kept in a chamber at 5°.

The performance on the diet containing the whole potato was again the best, the average weekly weight increase over a period of 6 weeks was 10.1 g, the ratio weight increase (g)/N eaten (g) during 3 weeks was 9.5, the coefficient of digestibility 79.2. The corresponding figures for the rats receiving their N from tuberin were 10.5 g, 8.3 and 56.4%, and for those receiving it from the combination of tuberin and pulp 8.6 g, 6.8 and 47.5%. The above values for the economy of utilization of the N are certainly influenced by the low digestibility of the N in the tuberin and the pulp.

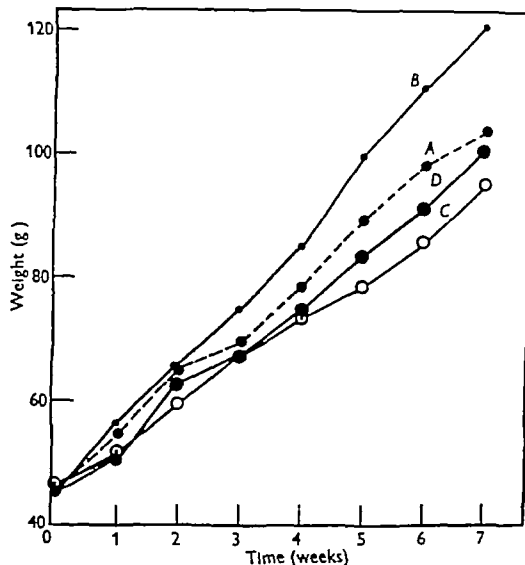


Fig 6, Exp 6 Average weight curves of five rats maintained from weaning on diets containing 1.74-1.82% N (on dry wt) derived from potato sample KE 10. A, whole potato steamed and dried with skin (diet 107), B, whole potato dried without skin (diet 107A), C, $\frac{2}{3}$ from crude tuberin, $\frac{1}{3}$ from unwashed pulp, $\frac{2}{3}$ from protein free press juice (in approximate proportions as present in the whole potato, diet 108), D, $\frac{1}{3}$ from tuberin, $\frac{2}{3}$ from protein free press juice (diet 109).

In Exp 6 the separation of the materials was effected as quickly as possible to minimize any changes that might follow this interference with the tissues. Neither the protein separated from the sap, nor the residual pulp, was washed, both were quickly dried at low temperature immediately after separation. In order to make the comparison with the whole potato more exact, tests were made with potatoes dried without removal of the skin (diet 107), seeing that in the preparation of the pulp the skin of the potato was included. The composition of the diets fed to the different groups of rats is set out in Table 6. In diet 108 the fractions of the N contributed by the tuberin ($\frac{1}{3}$), the protein free juice ($\frac{2}{3}$) and the pulp ($\frac{1}{3}$), were arranged to reproduce approximately the fractions contributed by these constituents in the whole potato. The average weekly weight increase (6.9 g) of the rats on diet 108 over the 7 weeks of the test was, however, significantly less than that of the animals on diet 107 receiving their N from the whole potato with its skin (8.2 g). In diet 109 the N was contributed equally by the crude tuberin and the protein free juice, an analysis of the

whole dried potato by Stutzer's (1880) method having shown that in this sample about 50% of the N was in the form of protein. On this diet the average weekly weight increase was 7.9 g, a value which should be compared with that of 10.8 g achieved on the diet containing the whole potato after the removal of the skin (see Fig. 6).

The values for Exp. 6 in Table 7, which refer only to the period of 2 weeks during which measurements of the food and N intake and of the digestibility of the N were made, show the lowered degree of digestibility and of utilization of N when the pulp was included in the diet. The mixture of protein and N.F.N. in diet 109 also functioned less efficiently than the mixture in the whole potato after removal of the skin (diet 107A), with which in this case the comparison should strictly be made. Nevertheless, the results show clearly the existence of a nutritionally advantageous interaction between the protein and N.F.N. of this diet.

In all the above tests the N in the skinned whole potato was more economically used than any other fraction or combination of fractions except, possibly, the sample of tuberin tested in Exp. 4. Some possible explanations of this fact are discussed below.

DISCUSSION

The main experimental findings of the present work are summarized in Tables 1, 3, 5 and 7, and it is mainly around these results that the discussion is centred. One conclusion that may immediately be drawn concerns the extreme lability of the nitrogenous constituents of the potato, both as regards their chemistry and their nutritive value. The deterioration in nutritive value on storage of the potatoes tested in Exp. 1a-c (Table 3) is obviously in keeping with the changes in chemical composition reported on p. 212. From these findings alone it is obvious that any attempt to collate a series of results obtained on such labile material over a period of some years will meet with considerable difficulty. The task is made less easy when it is realized that the mildest chemical manipulations of the experimental material may produce changes profoundly affecting the nutritive value of its nitrogenous constituents. The potato tuber is a living, changing system and the processes involved in the separation of its constituents, no matter how carefully operated, seem to bring about unknown changes detrimental to nutritive value. Thus in Exp. 6 the nitrogen of a recombination of tuberin, protein free juice and potato pulp had a nutritive value appreciably lower than that of the corresponding whole potatoes from which they had been prepared, the digestibility of the nitrogen of the reconstituted potato was only 69.2 as compared with 76.3% for the nitrogen of the whole potato.

The digestibility coefficients of the nitrogen of the preparations of tuberin used throughout the series of experiments were lower than that of the whole potato nitrogen, the mean value being 63% (range 56-70%) as compared with the mean value of 76%

(range 75-79%) for whole potato. Nevertheless, it seems doubtful whether a difference in digestibility of this order can entirely account for the equal or superior nutritive value of the mixture of protein and non-protein nitrogen in the whole potato compared with that of tuberin.

The complementary action in nutritive effect between the protein and non-protein nitrogen in the potato is clearly shown in the present work. In Exp. 3, the non-protein nitrogen in the expressed sap was shown to possess no growth supporting capacity when incorporated in a diet to the extent of 2% on dry weight. Nevertheless, when one-quarter of this nitrogen was replaced by tuberin nitrogen, in a diet containing 1.7-1.8% nitrogen, growth was promptly initiated at a rate not inferior to that supported by a similarly composed diet containing nitrogen derived solely from tuberin (see Fig. 3). A similar result can be derived from Exp. 4, where rats, which received their supply of nitrogen as the mixture of protein and non-protein nitrogen present in the expressed sap (diet 36B), gave a performance of the same order as that of litter mates whose diet (39) contained a similar proportion of nitrogen from tuberin (see Table 7 and Fig. 4).

No supplementary action could be demonstrated between tuberin and any insoluble protein remaining in the potato pulp, either when these two fractions were fed directly in combination as in Exp. 5 or compared indirectly as in Exp. 2. In the latter experiment the removal of the skin and outer cortex, which are the parts richest in the insoluble protein, increased rather than decreased the nutritive value of the remainder of the potato.

The results of amino acid analyses of tuberin and protein free juice do not provide an explanation for the complementary action between these two fractions. From the results given in Table 1 it is clear that any replacement of tuberin nitrogen by nitrogen from protein free juice would lead to a reduction in the amount of ten essential amino acids present. Nor, unless some insoluble protein which could not account for more than 7-10% of the total potato nitrogen were present in potato pulp, and were exceptionally rich in these essential amino acids, could the whole potato contain more of these than is contained in a corresponding amount of tuberin.

Two explanations of this conflicting series of findings are possible. (1) The feeding experiments with separated potato fractions may be vitiated by chemical changes taking place during separation of the fractions, thus making a fair comparison with the intact potato impossible. (2) The potato may contain substances, other than proteins and amino acids, which remain undetected by the usual chemical methods of examination, but which are capable of exerting a supplementary or sparing effect upon protein.

A recent paper by Rose, Oesterling & Womack (1948) contains a description of experiments in which growth of weanling rats was definitely stimulated by addition of 2 % glutamic acid to a diet in which the nitrogen, adequate in amount, was supplied as a mixture of the ten 'essential' amino acids. It is therefore possible that some nutritional significance attaches to the relatively large amounts of glutamine present in the potato tuber. In the sample of King Edward potatoes, of which the analysis is given in Table 1, 11.4 % of the total nitrogen and 24 % of the non protein nitrogen were present as glutamine, the corresponding values for the content of free amino acids being 10.1 and 21.5 % respectively.

SUMMARY

1 In the variety of potato studied, King Edward grown in the Fenlands, the total nitrogen varied from 1.7–2 % on dry weight, the proportion present as protein varied from about 40 to 50 % and was slightly lowered when the potatoes were stored. In the expressed juice of the raw potatoes about 30 % of the nitrogen was protein nitrogen of the non-protein nitrogen about one fifth was present in free amino acids, two fifths in amides and two fifths in nitrogenous bases. After extraction of the residual pulp with salt solution and alkali, 7–10 % of the total nitrogen remained unextracted.

2 At least two soluble proteins were detected in the press juice after deposition of the particles of the starch, one (α globulin) precipitated from the diluted sap on standing at pH 4, the other (β globulin) on boiling the filtrate therefrom. In one specimen of freshly dug potatoes the ratio of α - to β globulin was about 1/2. In a sample of stored potatoes it was about 2/1.

3 Both the nutritive value, judged by capacity to support growth in weanling rats, of the nitrogen in the whole steamed potato and the economy of its utilization, were less than those of the nitrogen in potatoes deprived of the skin and still lower than those of the nitrogen in peeled potatoes from which a layer of 2 mm thickness had been removed.

4 The growth promoting value of the potato nitrogen was usually found to be diminished after storage, the extent of deterioration depended on the

conditions, being least after storage at 5°. The ratio of protein to non protein nitrogen was also lowered after storage.

5 On the basis of nitrogen content, the nutritive value of tuberin prepared from the sap by heating at 80° at pH 4.0 was not superior to that of the mixture of protein and non-protein nitrogenous material in the whole potato. The non protein nitrogen in the press juice, after removal of the tuberin, was unable to support growth, but when combined with tuberin in the proportions (56/44) in which non-protein and protein nitrogen existed in the whole potato, the nutritive value was raised to a level approximating to that of tuberin, although still inferior to that of the nitrogenous mixture *in situ* in the intact tuber. This result shows the existence of a complementary nutritive action between the protein and non protein nitrogen in the potato.

6 The coefficient of (apparent) digestibility of the nitrogen was about 76 in the intact potato, about 65 for tuberin, 83 for the press juice and about 69 for the artificially reconstituted potato. The relatively low digestibility of the nitrogen in the separated protein, and of that in the mixture of this with the pulp, may partly account for the inferior nutritive value of the artificially reconstructed potato compared with the original it was designed to simulate.

7 The supplementary nutritive effect between the tuberin and the protein free fraction of the press juice could not be explained in terms of their amino acid contents. Nor, unless the potato contains an insoluble protein richer in essential amino acids than is tuberin, should the whole potato furnish these amino acids as abundantly as can tuberin. The somewhat inferior value of tuberin, compared with that of the mixture of nitrogenous substances in the intact tuber, may possibly be due to chemical degradation during its separation.

We desire to thank Sir Charles Martin, F.R.S., for his encouragement and helpful criticism during the course of this work and for his hospitality at Roebuck House, Chesterton, Cambridge, to which, at his invitation, the Division of Nutrition of the Lister Institute was evacuated during the war years. We are also indebted to Mrs M. E. M. Cutting for help in carrying out Exp. 3 and to Mr G. W. Flynn for assistance in the care of the experimental animals.

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The Pectic Enzymes of the Mould *Byssoschlamys fulva*

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(Received 28 February 1949)

The fungus *Byssoschlamys fulva* was discovered by Olliver & Smith (1933), and its action on pectic substances first studied by Olliver & Rendle (1934) Hurst & McMaster (1932-3), Hull (1933-4, 1934-5, 1939) and Gillespy (1936-7, 1939, 1940, 1946) have described its characteristics, while its metabolic products have been investigated by Raistrick & Smith (1933). It derives its economic importance from three characteristics, two of which are unusual or even unique (a) its ascospores have an abnormally high heat resistance and can readily withstand the normal processes used in bottling fruit, though only a small proportion survive the higher temperatures used in canning, (b) it can grow under conditions of greatly reduced oxygen tension, e.g. in canned and bottled fruit, and (c) it secretes pectic enzymes which can cause disintegration of the fruit tissue. Ascospores of *B. fulva* are thus liable to survive the usual bottling processes and cause more or less complete breakdown of the fruit.

The present work is an attempt to identify the particular pectic enzymes involved. The nomenclature used in this paper is in conformity with current views on the structure of pectin (Hirst & Jones, 1946), which support the classification of pectic enzymes proposed by Kertesz (1936) (1) protopectinase (or pectosase) which converts protopectin into soluble pectin, (2) pectin methoxylase (or pectase) which converts pectin into pectic acid and methanol, and (3) pectinase (probably a group of enzymes) which causes a reduction in the molecular size of the pectin and leads ultimately to D galacturonic acid. The group contains at least a disaggregating enzyme and polygalacturonase

B. fulva, the fruit juice being separated from the disintegrated fruit tissues by repeated filtration through cotton wool, (2) Czapek Dox solution on which a pure culture of the mould had been grown, (3) extracts of the fungal mycelium collected from various types of culture solution. More concentrated enzyme preparations were obtained from each of these sources by the addition of ethanol (3 vol to 1 vol of solution) and separation of the flocculent precipitate in a centrifuge. The solid product was freed from ethanol by drying over CaCl_2 in an evacuated desiccator and then dissolved in a small volume of water. The solution was kept sterile by storage at low temperature over CHCl_3 .

Substrates Potato disks of standard dimensions (1.2 cm diam, 0.5 mm thick) prepared on a simple hand microtome were used for the protopectinase tests. Pectin solutions (prepared from citrus pectin, 100 grade, British Drug Houses Ltd) and polygalacturonide methyl ester, prepared by the action of methanolic HCl on pectin (Morell, Baur & Link, 1934) were used for the pectin methoxylase and pectinase tests. Pectic acid, prepared by demethoxylating pectin with pectin methoxylase from tomatoes, was also used for some of the pectinase tests.

Methods employed for enzyme detection (a) Protopectinase was detected by its macerating action on thin disks of potato tuber at 37°. The disks were periodically removed from the enzyme solution and any maceration observed by gently pulling them with blunt-ended forceps. The results were placed on a semi quantitative basis by using a scale of softening ranging from 'just detectable' (+) to 'complete disintegration' (++++) (b) Pectin methoxylase activity was examined by Kertesz's (1937) method in which the carboxylic acid groups set free by the action of the enzyme are titrated with dilute alkali, using methyl red as indicator. (c) Pectinase activity is difficult to assess because of the complexity of the reactions involved. Periodic determinations of the viscosity and of the reducing power of pectin solutions on treatment with the enzyme preparation were chosen as measuring respectively the concentrations of unchanged substrate and the final decomposition product, D galacturonic acid.

Blank experiments with heat-inactivated enzyme solutions were included in all tests.

EXPERIMENTAL

Enzymes Three sources were used (1) bottled fruit which showed pronounced disintegration due to infection with

RESULTS

Protopectinase Protopectinase activity was detected in four out of seven cultures of *B. fulva* in Czapek Dox medium all containing 5% glucose, and in some cases, 0.05% asparagine. The most active culture gave + and ++ ratings in the potato disk test in 3 and 18 hr respectively. Two of those inactive were anaerobic cultures with submerged flocculent mycelia, in contrast to the thick white surface mycelia observed in aerobic cultures at pH 3-4. The single inactive aerobic culture was grown at pH 5 and formed a partly submerged mycelium similar to the anaerobic cultures. There was some indication that the addition of asparagine to the culture medium enhanced the production of protopectinase.

Protopectinase activity was detected in five out of six specimens of bottled fruit (plums, damsons, gooseberries) showing partial or complete disintegration of the fruit tissue. The most active specimen gave a potato disk rating of ++ in 6 hr, the remaining four showing +++ in 24 hr.

Activity tests over the pH range 2-8 were made with filtered fruit juice, the pH of the juice being adjusted with N acid or alkali. Maximum activity was observed at pH 4-5 (cf Davison & Willaman, 1927, Brown 1915, who gave pH 5 and 6 respectively).

Experiments on the thermal inactivation of the enzyme at pH 4 are shown in Table 1. The results indicate that the enzyme is completely inactivated in a medium of pH 4 by exposure to a temperature of 65° for 5-10 min.

Table 1 *Effect of heat on protopectinase of Byssoschlamys fulva*

Temp (°)	Time of heating (min)			
	5	10	15	20
	Activity rating (see test)			
50	++	++	++	++
55	++	++	++	++
60	++	++	++	+
65	±	-	-	-
70	-	-	-	-

Pectin methoxylase This enzyme was consistently absent from *B. fulva* culture media, from extracts of the fungal mycelium, and from the juice of infected bottles of fruit. The specimens tested were the same as those used in the protopectinase tests.

Control experiments with tomato juice and the commercial pectic enzyme preparation known as 'Pectinol 10 M,' using 1% pectin solutions as substrate, showed that Kertesz's (1937) method is only suitable for the qualitative demonstration of pectin methoxylase in an enzyme system. Quantitative tests are made difficult by an apparent dependence

of the activity of the enzyme sample (in 'Kertesz units'/ml), on the size of the sample and the duration of the test. In a typical experiment a 1% pectin solution (40 ml), which had been adjusted to pH 6.5 with 0.1 N sodium hydroxide using methyl red as indicator, was treated with 1 ml of a 'Pectinol 10 M' solution containing 0.2 mg of the enzyme; acid was formed in the solution in 2.5 hr at 15°. In similar experiments, using *B. fulva* enzyme preparations in various concentrations (1-5 ml of the concentrated solution in 40 ml of 1% pectin solution), no acid was produced, even after 5 days. Further experiments at 30° again pointed to the absence of pectin methoxylase activity in the *B. fulva* enzyme system, whereas at this temperature 'Pectinol 10 M', in the same concentration as before, produced acid within 1 hr.

Independent pH measurements using a Cambridge pH meter confirmed the failure of all the *B. fulva* enzyme preparations to produce acid from a pectin substrate.

Pectinase complex Pectin solutions which were treated with *B. fulva* enzyme preparations at 25 and 37° showed a rapid initial fall in viscosity, followed by a slower fall to a constant value (see Fig 1).

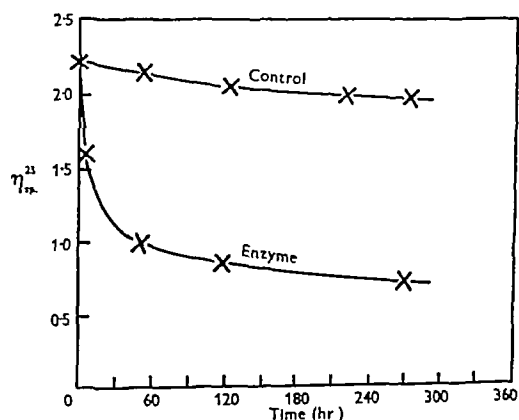


Fig 1 Effect of *B. fulva* enzymes on the viscosity of 1% pectin solution at 25°

Simultaneous determinations of the reducing power by the alkaline iodide method (Willstätter & Schudel, 1918, Ingles & Israel, 1948) indicated that only a minute increase took place which was of the same order as the experimental error of the estimation (0.05 ml of 0.1 N iodine). Typical results are shown in Table 2.

The results obtained over the pH range 3-5 were quantitatively similar and an optimum pH value was not apparent.

Control experiments with 'Pectinol 10 M' at 37° indicated that the reducing power of 0.4% pectin solution did not increase until the specific viscosity of the solution had fallen to a value of approxi-

mately 0.2. Further tests were thus made with a *B. fulva* enzyme preparation in which the pectin solutions were held for long periods at 37° after the viscosity had reached a constant value ($\eta_{sp}^{25} = 0.12$). Even 1000 hr after the viscosity of the pectin solution had become constant, the increase in reducing power remained negligible. On the other hand, 0.4% pectin solution (40 ml) which had been treated with 0.2 mg of 'Pectinol 10 M' at 37° rapidly gave an increase in reducing power equivalent to 0.29 ml of 0.1 N iodine/2 ml of pectin solution.

Table 2 *Effect of Byssochlamys fulva enzymes on the viscosity and reducing power of pectin*

Pectin (%)	Mixture	Temp (°)	Viscosity (η_{sp}^{25})		Reducing power of 2ml mixture (ml 0.1 N I ₂)	
			Initial	Final	Initial	Final
1.0	Test	25	2.22	0.70	0.13	0.15
1.0	Blank	25	2.22	1.99	0.10	0.13
0.4	Test	37	1.31	0.12	0.33	0.38
0.4	Blank	37	1.31	1.02	0.34	0.30

The material recovered by precipitation with ethanol from the *B. fulva* test and from blank solutions after the viscosities had become constant showed markedly different physical properties. The precipitate from the test solution was non-gelatinous and flocculent, whereas that from the blank solution was gelatinous and voluminous, resembling undegraded pectin precipitated under the same conditions.

A 1% polygalacturonide methyl ester solution ($\eta_{sp}^{25} = 0.27$) was treated with an extract of *B. fulva* mycelium which rapidly disaggregated pectin. The viscosity of the solution did not fall in 300 hr and the reducing power of the polyester was similarly unaffected.

In view of the fact that Jansen & MacDonnell (1945) have stated that only de-esterified pectic substances are attacked by polygalacturonase, a suspension of pectic acid in water was treated with the *B. fulva* enzymes. Failure to obtain any increase in reducing power further confirmed the absence of polygalacturonase from the *B. fulva* enzyme system.

DISCUSSION

The present results show that *B. fulva* produces an enzyme system possessing protopectinase and pectinase activity. Protopectinase activity is found in many fungi and bacteria (Branfoot, 1929) and it has long been recognized that the macerating action of many plant pathogens is due to the production of protopectinase and the consequent destruction of the middle lamella. Protopectin, the substrate for

this enzyme, is ill defined so that work on this enzyme has been mainly qualitative.

Although little attention has been paid to the individual components of the complex, the pectinase group of enzymes appears to contain at least polygalacturonase and a disaggregating enzyme. Many workers have obtained pectinase preparations with very marked polygalacturonase activity, thus Ehrlich (1933, 1935), Mottern & Cole (1939), and Manville, Reithel & Yamada (1939) have broken down pectin completely into D-galacturonic acid. On the other hand, Kertesz (1939) has observed that many pectinase preparations from various *Penicillia* bring about the development of only a small fraction of the maximum possible reducing power from pectin. This variation in activity may be due to the existence of two or more polygalacturonases, acting on pectic substances of various molecular sizes, so that all the enzymes are necessary to bring about the complete sequence of reactions required to produce D-galacturonic acid. Kertesz (1939) has suggested that two different types of enzyme are involved in pectinase activity, the first disaggregating the pectin molecule, with a consequent fall in viscosity, and the second attacking the product so formed, opening the glycosidic bonds between the uronic acid residues with a consequent increase in reducing power.

The results of the present investigation indicate that a very large reduction in the molecular size of pectin is brought about by the enzyme system of *B. fulva*, without any increase in reducing power, and therefore without any fission of glycosidic bonds. This result is an extreme case of the phenomenon noted by Kertesz (1939) amongst the pectinases of various *Penicillia*. It is considered that the results provide confirmatory evidence for Kertesz's view that the large molecular size of pectin is mainly due to the existence of aggregates, consisting of relatively small polyuronic acid units joined by linkages which are not glycosidic but possibly of the nature of hydrogen bonds.

SUMMARY

1. The enzyme system produced by the mould *Byssochlamys fulva* has been examined. Evidence has been found for the presence of protopectinase and a disaggregating enzyme which reduces the molecular size of pectin without the production of galacturonic acid.

2. The production by a fungus of a pectinase type of enzyme which is free from pectin methoxylase has not been reported hitherto.

The authors would like to express their thanks to Mr W. B. Adam and Dr T. G. Gillespy for their interest and advice.

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Studies on Suramin (Antrypol: Bayer 205)

7 FURTHER OBSERVATIONS ON THE COMBINATION OF THE DRUG WITH PROTEINS

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(Received 28 February 1949)

Little is known about the metabolic fate of injected suramin, the trypanocidal drug which is retained in the body for several weeks or even months after its injection. Even before satisfactory methods for the determination of suramin became available, however, it was suggested that the injected drug combines with the body proteins (for a review of the early literature see Findlay, 1939), and this view received support from the observation that the addition of suramin effects marked changes in some of the properties of certain proteins, for example, the serum proteins are protected against heat coagulation (Collier, 1926, 1927) and against precipitation by tannin, mercuric chloride and certain other reagents (Jirovec & Kocian, 1930, Kocian, 1936), the isoelectric points of some immune substances are altered (Klopstock, 1932), and the drug inhibits a few enzymes, e.g. trypsin (Beilinson, 1929), fumarase (Quastel, 1931) and hyaluronidase (Beiler & Martin, 1948).

Quantitative measurement of the amount of suramin which combines under varying conditions with the plasma proteins of animals injected with the

drug, or in experiments *in vitro* with various proteins, became possible when a satisfactory chemical method became available for the determination of the drug (Dangerfield, Gaunt & Wormall, 1938). Using this method it was conclusively shown that suramin can combine with many different proteins (serum globulin, fibrinogen, casein, gelatin) under physiological conditions of pH and temperature (Boursnell & Wormall, 1939, Dewey & Wormall, 1946).

At present we are investigating the action of suramin on enzymes. It was thought, therefore, that a study of the conditions under which the drug combines with proteins, and a further investigation of the nature of the linkages concerned, might throw some light on the specific inhibitory effect which the drug exerts on some enzyme systems. It was also hoped that useful information might be obtained about the mechanism whereby suramin inactivates complement and the blood clotting system.

The present paper describes a study of (1) the stability of the linkage between the drug and various proteins, (2) the influence of pH on the combination,

(iii) the failure of suramin to combine, *in vivo* and *in vitro*, with the proteins of the intact red blood cell, (iv) the fate of intravenously injected suramin-protein complexes and (v) the protein groups which might be concerned with the binding of suramin

METHODS AND MATERIALS

Suramin determinations These were carried out as described previously (Dangerfield *et al* 1938, Boursnell, Dangerfield & Wormall, 1939, Dewey & Wormall, 1948) The values for rabbit plasma have been corrected for the small amount of amine precursor (equivalent to about 1.0 mg suramin/100 ml.) present in normal plasma. A similar correction has been made for the precipitated suramin-protein complexes, but this blank value is usually negligible (equivalent to 0.01–0.02 g suramin/16 g protein N)

Total N determinations were carried out on most of the precipitated suramin-protein complexes, and the suramin contents of the complexes (except those referred to in Table 1) are given as g of suramin/100 g of the complex, allowance being made for the N content of suramin (5.88%). The N content of the untreated proteins was assumed to be 16% for the mixed serum proteins and 15.76% for crystalline egg albumin (Chibnall, Rees & Williams, 1943). This method of recording the results was adopted since the methanol-precipitated complexes contained appreciable amounts of inorganic material.

Injections The solutions of suramin or suramin-protein complexes, in 0.9% (w/v) NaCl, were injected into the marginal ear veins of rabbits, and blood samples were taken from the ear not used for the injection and collected in 'oxalated' tubes. The plasma was then separated as quickly as possible.

Amino N determinations These were made by titration from pH 7 to 9, with a further titration back to pH 9 after the addition of excess of neutralized 20% formaldehyde solution. (For full details see Boursnell, Francis & Wormall, 1946)

Buffer solutions The 'Universal' buffer (Baird and Tatlook (London), Ltd.) of Johnson & Lindsey (1939) was used for the range pH 3–9, and the sodium acetate-HCl buffer of Walpole (cf. Vogel, 1944) for pH 1. For some experiments a 0.75M-NaHCO₃-H₂CO₃ buffer of pH 7.5 was used.

Egg albumin This was thrice crystallized hen ovalbumin

RESULTS

The precipitation of suramin-protein complexes by dilute acid

It was found that mixtures of suramin and serum gave heavy precipitates when the solutions were made slightly acid, the pH of maximum precipitation varying with the protein used and the amount of suramin added. These precipitated complexes contained much suramin, and it was decided to determine whether repeated solution and precipitation of these complexes would alter their suramin content. In a typical experiment the drug-protein complex was precipitated from a mixture of suramin and human serum by adjusting the pH to about 4.0,

followed by solution in 0.9% NaCl at pH 7.5, and a repetition of the precipitation a further seven times. Analysis of the precipitates, some washed with 0.9% NaCl and some washed with methanol, showed that there was very marked consistency in the suramin content of the complexes during this serial precipitation, even up to the eighth precipitation (Table 1). The methanol-washed precipitates contained slightly less suramin than did those washed with NaCl, but in this case also there was no fall in the suramin content of the complex during five precipitations.

Table 1 *Suramin content of acetic acid precipitated suramin-protein complexes from a mixture of suramin and human serum*

(Suramin, 1.0 g dissolved in 5.0 ml of 0.9% NaCl, was added to 150 ml of human serum and the mixture kept for 1 hr at room temperature. The suramin-protein complex was then precipitated by the addition of N HCl and finally 2N acetic acid to give maximum precipitation (pH about 4.0). Suramin determinations were made on part of the precipitate and the rest was dissolved in 0.9% NaCl with the addition of a little 0.75M-Na₂CO₃-H₂CO₃ buffer of pH 7.5. Precipitation and solution were repeated a further seven times and suramin determinations were made on samples of the precipitates washed (a) twice with 0.9% NaCl, or (b) twice with methanol)

No of precipitations	Suramin content of the precipitates (%)	
	NaCl washed	Methanol extracted
1	13.4	11.8
2	12.4	11.9
3	13.5	12.4
4	13.8	11.8
5	13.3	13.1
8	12.8	—

In other experiments egg albumin solutions were treated with varying amounts of suramin, the concentration of the drug in the mixture ranging from 0.21 to 1.67% (i.e. 1.5–11.7 × 10⁻³M). The drug-protein complexes were precipitated by adding acetic acid, and after five precipitations contained 10.74–17.54 g of suramin/100 g of the drug-protein complex (Table 2). The amount of drug bound by the protein in the mixture with the lowest concentration of suramin used in this experiment was surprisingly high, it was 10.74 g suramin/100 g of the drug-protein complex, this being equivalent to the combination of 12.0 g of suramin with 100 g of protein, or 3.7 suramin groups/molecule of egg albumin, assuming a molecular weight of 44,000 for this protein.

The product obtained from the mixture with the highest concentration of suramin used in this experiment contained 17.54 g of suramin/100 g of the drug-protein complex. This is equivalent to the

combination of about 21 g of suramin with 100 g of egg albumin, or the average introduction of 6.5 suramin groups into each protein molecule

Table 2 *Combination of egg albumin with varying amounts of suramin*

(Varying amounts of a 10% (w/v) solution of suramin were added to samples of a 2.36% solution of crystalline egg albumin and the mixtures kept at about 14° for 30 min. The suramin protein complexes were then repeatedly precipitated by the addition of 5N acetic acid, and dissolved in water at about pH 7.5, five precipitations in all.)

Egg albumin solution (ml)	Suramin solution (ml)	Water (ml)	Suramin content of precipitated complex	
			(g/g total N)	(g/100 g suramin protein)
20	0.5	3.5	0.73	10.74
20	2.0	2.0	1.18	16.65
20	4.0	0	1.25	17.54

Influence of pH on the combination of suramin with proteins

Suramin undoubtedly combines firmly with serum and other proteins at pH 7.5, but the amount of suramin combining at that pH is not necessarily the same as that present in the suramin protein complexes precipitated from the same solution by the addition of acid. Experiments were therefore made to determine the amount of suramin combining with proteins at different pH's. The drug protein complexes were precipitated with methanol, without materially altering the pH of the mixture, and the precipitates were washed with methanol, in which free suramin is very soluble.

N HCl or 0.1N NaOH was added to rabbit serum (2 ml.) to give approximately the pH required. Buffer solution was added, together with 0.9% NaCl to give a total volume of 5.5 ml. The suramin solution (1.0 ml. of a 3% (w/v) solution in 0.9% NaCl) was then added to each tube, and the mixture was shaken and kept at room temperature for 1 hr. with occasional shaking. Methanol (26 ml.) was then added to each mixture, and the centrifuged precipitates were washed four times with methanol (7 ml. each time), and dried *in vacuo* over CaCl₂.

The results of this experiment (Table 3) showed that under these conditions the serum proteins combined at pH 1 or 3 with about twice as much suramin as they did at pH 5. At pH 7 the amount of combined suramin was only about one quarter, and at pH 9 about one fifteenth, that combined at pH 5. A similar experiment was carried out with egg albumin over the same pH range, but with additional mixtures at pH's 6 and 8.

The solution of crystalline egg albumin (2 ml. of 5.8% w/v) was brought to the required pH and mixed with buffer, 0.9% NaCl and suramin solution as in the above experiment

with rabbit serum proteins. The suramin protein complexes were precipitated and washed with methanol and were then dried and analysed.

The results (Table 3) were similar to those obtained with the serum proteins, they showed that much more suramin combines with protein in the more acid solutions used in these experiments.

Table 3 *The influence of pH on the combination of suramin with normal rabbit serum proteins and with egg albumin*

(See the text for experimental details. Each experiment was carried out in duplicate and the two sets of results are recorded below.)

pH	Suramin content (g/100 g suramin protein complex) of the methanol precipitated complexes of suramin and	
	Rabbit serum proteins	Egg albumin
1	22.9, 22.7	20.95, 20.95
3	22.6, 22.5	19.7, 18.7
5	11.9, 10.6	4.6, 5.2
6		0.63, 0.76
7	2.8, 2.5	0.49, 0.59
8		0.28, 0.34
9	0.80, 0.64	0.31, 0.23

Combination of suramin with the proteins of red blood cells

Earlier investigations (Dewey & Wormall, 1948) have shown that suramin added to whole blood, or to suspensions of red blood cells, does not pass into the red cell. This might be due to inability of the drug to pass through the membrane, or to an inability of suramin to combine with haemoglobin and the stroma protein.

Although suramin did not enter the red cell in these *in vitro* experiments it does not follow that the same will hold true *in vivo*. Experiments have therefore been made to determine whether the drug enters the red cells after it has been intravenously injected into rabbits. Estimations were made on the separated red cells after they had been washed with 0.9% NaCl, and also on the unwashed cells (to exclude the possibility of loss of suramin by the cells during the washing process). In all these experiments, with plasma suramin values up to 69 mg/100 ml, no significant amount of the drug was found in the red cells, the very small amount occasionally found in the unwashed red cells was not more than could be accounted for by the presence of a small amount of suramin-containing plasma in the cell fractions.

The second possibility mentioned above was examined by determining the capacity of haemolysed red cells to combine with suramin. In one group of experiments, the results of some of which are given in Table 4, the red cells of normal rabbit blood were washed with 0.9% NaCl, haemolysed

Table 4 *Combination of suramin with the plasma and cell proteins of normal rabbit blood*

(The suramin solution (2.8 of 10% (w/v) in 0.9% NaCl solution) was added to the mixture of plasma and 0.9% NaCl, or to the haemolysed red cell solution. Each mixture was kept at room temperature for 15 min with occasional shaking, it was then treated with an equal volume of 0.9% NaCl solution and the protein suramin complex precipitated by the addition of 2N acetic acid. The precipitate was dissolved in 3 ml of 0.9% NaCl solution with the aid of sufficient 2N Na₂CO₃ to give pH about 7.5 and the precipitation with acetic acid was repeated a further five times.)

Exp no		Plasma or haemolysed cells* (ml)	0.9% NaCl (ml)	Suramin solution added		Suramin content of precipitated suramin protein complex	
				(ml)	(mg suramin)	(g/g total N)	(g/100 g complex)
1	Plasma	1.5	1.5	0.5	14	0.73	10.9
		1.5	1.5	1.0	28	0.96	14.0
	Haemolysed cells	3.0	0	0.5	14	1.13	16.2
		3.0	0	1.0	28	1.47	20.5
2	Plasma	3.0	5.0	1.0	100	1.88	25.3
		3.0	5.0	2.0	200	2.03	26.9
	Haemolysed cells	8.0	0	1.0	100	1.72	23.4
		8.0	0	2.0	200	1.89	25.4

* Centrifuged cells from 1 vol of oxalated rabbit blood were washed well with 0.9% NaCl and then haemolysed with 4.5 vol of distilled water.

with water, and then mixed with a suramin solution, parallel experiments being made with the blood plasma. The mixtures of suramin and haemolysed red cells gave heavy precipitates when dilute acid was added and these complexes, reprecipitated a further five times, had a suramin content which was of the same order as that of the complexes separated similarly from the corresponding suramin plasma mixtures. For example, in Exp 2, where the plasma and haemolysed cells were diluted so that each solution contained the same amount (0.38%) of protein N before the addition of the suramin solution, there was a very close similarity between

the suramin contents of the complexes from suramin plasma mixtures and those from mixtures of suramin and haemolysed red cells.

The retention of injected suramin protein complexes

Experiments have been made to determine whether intravenously injected suramin protein complex is retained longer in the blood of a rabbit than is an equivalent amount of the free drug. In one experiment the drug was attached to crystalline egg albumin, and in the other rabbit serum proteins were used to form the complex, in the hope that this would

Table 5 *The suramin content of the blood plasma of rabbits following the intravenous injection of (a) suramin and (b) suramin protein complexes*

(For experimental details see the text.)

Exp no	Material injected	Suramin injected (mg/kg)	Suramin content of plasma (mg/100 ml) after					
			17 min	45 min	3 hr	24 hr	48 hr	7 days 14 days
1	Suramin	14.2		20.0	12.0	4.2	3.2	1.6
		23.3*	33.4		14.8	7.5	4.8	1.8
	Suramin rabbit serum protein complex	13.05		21.5	8.1	1.8	1.5	1.0
		22.0*	46.3		26.6	7.9	5.5	3.0
2	Suramin	21.9	46.5		19.8	6.4	4.4	2.4 1.6
			39.2		15.2	5.9	4.0	1.9 1.6
			42.0		16.4	6.6	4.3	2.3 2.0
	Suramin egg albumin complex	{ 21.8 (a)† 22.1 (b)†	41.3		15.8	7.0	5.6	1.6 1.7
			37.2		15.5	5.9	4.1	1.6 1.1
			37.0		12.8	5.7	3.5	1.9 1.7
			36.6		15.3	6.2	3.4	2.1 1.6

* Given in two injections, with an interval of 36 min between them. The times of bleedings were reckoned from the second injection.

† (a) and (b) After it had been kept at 5° for a few days, the solution of suramin egg albumin complex prepared for this experiment contained a very small amount of solid matter. Two rabbits were injected with the uncentrifuged solution (b) and two with the clear centrifuged solution (a).

be less 'foreign' than the egg albumin complex and therefore less liable to suffer rapid breakdown in the rabbit

Exp 1 A suramin solution (300 mg in 10 ml of 0.9% NaCl) was added to a mixture of rabbit serum (15 ml) and 0.9% NaCl (15 ml), the mixture kept at about 13° for 30 min and the drug-protein complexes precipitated by the addition of 5N acetic acid to give maximum precipitation. After eight further precipitations with acid, the complex was dissolved in 0.9% NaCl (15 ml) at pH 7.0 and filtered through a Berkefeld filter. This solution, containing 0.88 g of suramin and 3.05 g of protein/100 ml, was injected into rabbits as follows: one rabbit (wt 2.7 kg) received 4.0 ml, and another rabbit (wt 3.0 kg) received 4.5 ml followed by a further injection (3.0 ml) 36 min later. Two control rabbits were injected under the same conditions with a solution containing 0.88 g of suramin/100 ml of 0.9% NaCl. Blood samples (about 7 ml) were taken from the rabbits at intervals for plasma suramin determinations.

Exp 2 Suramin (1.5 g in 15 ml of water) was added to a solution of crystalline egg albumin (150 ml containing 3.54 g of protein) and the drug-protein complex was precipitated by acid and purified by four further precipitations, as in *Exp 1*. The final solution (containing 1.26 g of suramin, 6.59 g of protein, 0.9 g of NaCl and 0.04 g of merthiolate/100 ml) was injected into four rabbits (one injection each), and three control rabbits received corresponding injections of free suramin.

The results of these experiments (Table 5) showed that the injected suramin-protein complex was not retained longer in the plasma than was an equivalent amount of the free drug. Individual rabbits occasionally show appreciable deviation from the average with regard to their retention of the injected drug or drug-protein complex, but in no case did any rabbit injected with the complex show any abnormally long retention of the suramin.

The combination of suramin with proteins treated with di-2-chloroethylsulphone

The combination of suramin with proteins might be due to a reaction between the sulphonic acid groups of the drug and basic groups of the protein. Tests were therefore made to determine whether the blocking of the free amino groups of the protein reduces its capacity to combine with suramin. Blocking of practically all the amino groups of gelatin can readily be effected at pH 8 and 30° by mustard gas sulphone, di-2-chloroethylsulphone (Banks, Bourns, Francis, Hopwood & Wormall, 1946), and the ability of the sulphone-treated proteins (gelatin and the serum proteins) to combine with suramin was, therefore, compared with that of the untreated proteins.

Exp 1 Gelatin (1.0 g) was dissolved in hot water (60 ml), the solution cooled a little and 0.75M NaHCO₃ (40 ml) added. The mixture was treated with di-2-chloroethylsulphone (0.5 g), stirred at 37° for 4 hr and then kept at 4° overnight. Next day a further 0.5 g of the sulphone was

added and the mixture stirred at 37° for a further 8.5 hr. The solution was then diluted with an equal volume of water and dialysed against distilled water for 24 hr at 4°. Total N and amino N (formaldehyde) determinations on this solution and on a dialysed 0.5% gelatin solution showed that the sulphone had blocked at least 95% of the free amino groups of the gelatin.

The dialysed gelatin and sulphone gelatin solutions (each containing 0.45% (w/v) of protein) were each treated with buffer solution and suramin (see Table 6 for details) and the mixtures kept at 18° for 1 hr. Each solution (36 ml) was then poured into ice-cold methanol (220 ml) and the precipitated complexes were centrifuged and washed three times with ice-cold methanol (25 ml each time). Mixtures C and D required the addition of a few drops of glacial acetic acid before the drug-protein complex could be precipitated by the methanol, and the methanol used for washing these precipitates was similarly acidified. The dried drug-protein complexes were dissolved in warm water (with the aid of a few drops of N NaOH in the case of C and D) and total N and suramin determinations were made on samples of these solutions.

Exp 2 Di-2-chloroethylsulphone (1.0 g) was added to a mixture of rabbit serum (24 ml) and 0.5M NaHCO₃ (24 ml) and the mixture stirred at 37° for 6 hr, N NaOH being added when necessary to maintain the pH between 7.5 and 8.5, after 2 days at 4°, the solution was again stirred at 37° for 6 hr after the addition of more sulphone (1.0 g). The mixed 'sulphone proteins' were precipitated by adding dilute acetic acid, dissolved at pH 7-7.5 and purified by three precipitations with N HCl. The final solution (20 ml) contained 4.86% protein, 0.9% NaCl and 0.02% merthiolate, total N and amino N determinations on this solution and on dialysed rabbit serum showed that 72% of the free amino groups of the serum proteins had been blocked by treatment with the sulphone.

Samples of the diluted solution of sulphone serum proteins were treated with varying amounts of suramin, as recorded in Table 7, and the mixtures were kept at 16° for 30 min, the drug-protein complexes were precipitated by addition of 5N acetic acid and purified by a further four precipitations. The final precipitates were dissolved in 0.9% NaCl with adjustment of the pH to 7.0, and total N and suramin determinations were made on these solutions.

The results show that mustard gas sulphone-treated gelatin combines with more suramin than does an equivalent amount of gelatin under similar conditions (Table 6). This increase may be partly due to the fact that these drug-protein complexes gave maximum precipitation at different pH's, but several other factors may also be involved. A similar increase in the capacity of the protein to react with mustard gas occurs when serum globulin is treated with sufficient phenyl isocyanate to block most of its free amino groups (Banks *et al.* 1946).

In other experiments it was found that the treatment of rabbit serum proteins with mustard gas sulphone (resulting in the blocking of about three quarters of the free amino groups of the proteins) did not reduce the capacity of the proteins to combine with suramin (Table 7).

Table 6 *Combination of suramin with gelatin and di 2 chloroethylsulphone treated gelatin*

(For experimental details see the text)

Protein		Vol. of protein solution (ml)	NaHCO ₃ , H ₂ CO ₃ buffer solution (pH 7.5) (ml)	Suramin (mg dissolved in 1 or 2 ml of water)	Suramin content of the methanol precipitated complexes	
					(g/g of total N)	(g/100 g complex*)
Gelatin	A	33	2	20	0.17	2.99
	B	33	2	40	0.26	4.54
Sulphone treated gelatin	C	33	2	20	0.22	3.69
	D	33	2	40	0.78	12.32

* Assuming 18.0% of N in gelatin and 17.18% N in the di 2 chloroethylsulphone treated gelatin

Table 7 *Combination of suramin with di 2-chloroethylsulphone treated rabbit serum proteins*

(For the experimental details see the text)

Sulphone treated serum protein solution* (ml.)	Suramin (mg dissolved in 0.2 ml of water)	Suramin content of precipitated drug protein complexes	
		(g/g total N)	(g/100 g complex)†
2.0	2.5	0.87	12.01
2.0	5.0	1.59	20.71
2.0	10.0	1.85	23.61

* Containing 1% (w/v) of protein in 0.9% NaCl solution.

† Assuming 14.89% N in the sulphone treated proteins (calculated on the basis of 16% of N in the mixed serum proteins and the reaction of the sulphone with 72% of the free amino groups of proteins)

DISCUSSION

The ability of suramin to combine with a variety of proteins under physiological conditions of pH and temperature is now well established, though the precise linkage by which the drug is firmly bound to the protein is still unknown. The reaction is rapid and although the mixtures of suramin and protein were usually allowed to stand for 30 min. or 1 hr. in the experiments described here, it is not suggested that this interval is necessary. Our enzyme inactivation experiments, a preliminary account of which has been given elsewhere (Town, Wills & Wormall, 1949), have shown that previous contact of drug and urease (and certain other enzymes) for 30 min. or so before addition of the substrate leads to an increased inhibition of the enzyme, but it is also true that the addition of suramin to solutions of serum proteins or egg albumin at pH 7.5 is followed by the immediate formation of drug protein complexes. These complexes can be precipitated by methanol or ethanol at pH 7.5 or by acidification to pH 4-5. The acid-precipitated complex undoubtedly contains more suramin than does the complex present at pH 7.5, but there is no doubt that an appreciable amount of the drug is firmly fixed to proteins at pH 7.5. Combination of the drug with the body proteins can account for the long retention of the injected drug,

and it seems probable that slow, continuous liberation of the free drug, possibly as a result of the hydrolysis of the protein of the complex, accounts for the relatively long protection against sleeping sickness which is afforded by one or more injections of suramin. The trypanocidal power of the drug protein complex is not known, but we hope to study this in collaboration with Dr F. Hawking.

The experiments described here show that suramin can combine as readily with haemoglobin, and possibly other proteins of the red blood cell, as it does with the plasma proteins. The failure of injected suramin to combine with the red cell is, therefore, due to inability of the drug to pass through the red cell membrane, presumably because of the size and/or spatial configuration of the suramin molecule. Suramin will not pass through relatively permeable collodion membranes (Boursnell *et al.* 1939) and in general it does not diffuse through animal membranes. Hawking (1940), for example, found no suramin, or faint traces only, in the cerebrospinal fluid of patients injected with the drug, even when the plasma contained as much as 17.2 mg of suramin/100 ml.

Our experiments with complexes of suramin and rabbit serum proteins (or egg albumin) have shown that retention of the drug in the blood following the intravenous injection of the preformed complex into a rabbit is not greater than that following the injection of an equivalent amount of uncombined suramin. These complexes will not, therefore, be of any special value for prophylactic purposes.

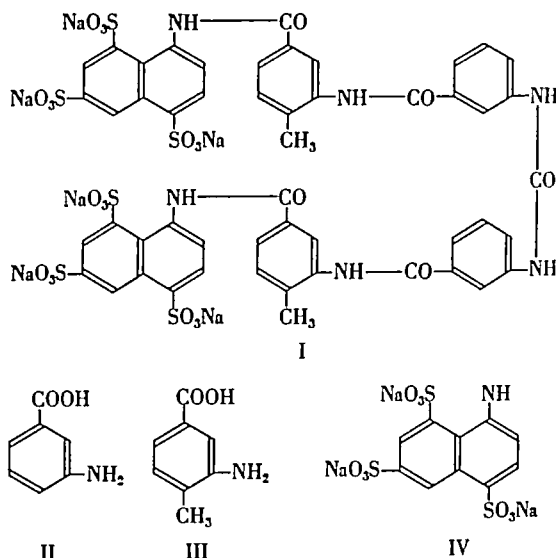
An appreciable amount of suramin combines with plasma proteins when the concentration of the drug is of the same order as that in the plasma after the injection of normal curative or preventive doses of 1 or 2 g of suramin/70 kg man. In some of our experiments, for example, we have found that the average plasma suramin values of rabbits after a single intravenous injection of 28 mg of suramin/kg were as follows: 74 (2 min after the injection), 69 (after 5 min), 62 (after 10 min) and 40 mg/100 ml (after 40 min). Protein suramin complexes containing nearly 1% of the drug were separated by methanol precipitation at about pH 7.5 from the

plasma of these animals shortly after the injections and also from egg albumin solutions (or normal plasma) to which suramin had been added to the extent of 80–100 mg/100 ml. Even with much lower concentrations of suramin there is an appreciable amount firmly combined with the plasma proteins. Thus the plasma of rabbits 3 and 26 hr after the injection of 28 mg of suramin/kg contained methanol precipitable protein drug complexes containing 0.24 and 0.08 % of suramin respectively.

The molecular weight of suramin is 1420, and the presence of a considerable amount of suramin in the complex does not require many suramin molecules/molecule of protein. In the experiments described in Table 2, for example, the complexes obtained contained 3.7–6.5 suramin groups/molecule of egg albumin (mol wt 44,000). The complexes obtained with rabbit serum proteins in a mixture containing 461.4 mg of suramin/100 ml at pH 7.0 (Table 3) contained the equivalent of about 2 mol of the drug/mol of serum protein (assuming an average mol wt of 100,000 for serum proteins). The complexes obtained in more acid solutions contain much larger amounts of suramin, and at pH 1 they corresponded to 21 mol of the drug/mol of rabbit serum proteins, and about 8.2 mol/mol of egg albumin. However, at pH 7.5 and with suramin concentrations similar to those found in the plasma of rabbits shortly after the injection of 28 mg of suramin/kg (equivalent to a dose of 2 g for a 70 kg man), the drug protein complexes contain much less suramin, the value corresponds usually to about 0.6 mol of suramin/mol of plasma protein.

Suramin (I) is a complex urea derivative containing three sulphonic acid radicals in each naphthylamine grouping. For several reasons it appeared probable that these naphthylaminesulphonic acid groups are responsible for the combination of the drug with proteins. We have found that acid-hydrolysed suramin, which contains *m* amino-benzoic acid (II), *m* amino *p* toluic acid (III) and 1 naphthylamine 4,6,8 trisulphonic acid (IV) (Lang, 1931), combines with proteins to produce acid precipitable products. Tests with the individual amines showed that of these three amines only (IV) is capable of forming a complex with proteins, this complex is apparently much less stable than is the corresponding suramin protein complex, since acid-hydrolysed suramin is not retained in the blood very long after its injection into rabbits (Dewey & Wormall, 1946). Furthermore, Spinks (1948) has shown that suramin analogues containing naphthalenepolysulphonic acids as end groups are generally similar to suramin with regard to their persistence in the blood after intravenous injection into rabbits, of the compounds of smaller molecular weight tested by this author, 1 naphthylamine 4,6,8 trisulphonic acid (IV) was fairly rapidly elimin-

ated after injection but was retained longer than was (II) or (III). Spinks concludes that 'marked persistence is a property of polyamides of high molecular weight that contain naphthylaminepolysulphonic acids as end groups'. From the evidence presented by Spinks and by ourselves, it seems fairly certain that the ability of suramin to combine with proteins is determined by its sulphonic acid groups.



Since the naphthylamine sulphonic acid groups of suramin appear to be concerned with this reaction, it seems most likely that they react with, or are bound by, basic groups of the protein. The results of our experiments suggest, however, that the free amino groups are not essential for the formation of suramin protein complexes, and it would appear that other basic groups are involved. Mandel & Steudal (1926) showed that suramin readily combines with protamines and histones, and we are making a more extensive study of this combination, and of possible interactions between suramin and various amino acid groupings, in the hope of gaining more information about the reaction between the drug and tissue proteins. A reaction with thiol groups can be excluded, since suramin does not appreciably reduce the thiol reaction of cysteine, reduced glutathione or heat coagulated egg albumin. Furthermore, although the drug strongly inhibits urease at pH 5.0, it is not a general poison for thiol enzymes (Town *et al.* 1949, Wills & Wormall, 1949).

SUMMARY

1 A further study has been made of the combination of the trypanocidal drug suramin with a variety of proteins.

2 Suramin-protein complexes are usually readily precipitated by the addition of dilute acid, the pH

for optimum precipitation depending on the relative amounts of the drug and the protein. Repeated precipitation by acid followed by solution at pH 7.5 does not reduce the suramin content of the precipitated complexes.

3 Considerable amounts of suramin become firmly attached to proteins such as the serum proteins and crystalline egg albumin at pH 7.5, but much more is bound by the protein in acid solutions, particularly at pH's below 6.

4 The absence of suramin from the red cells of animals injected with the drug is due to the inability of the drug to pass through the cell membrane. Suramin readily combines with the proteins of haemolysed red cells.

5 Suramin injected intravenously as a complex with rabbit serum proteins, or with egg albumin, is not retained longer in the blood than is the injected free drug.

6 The blocking of the free amino groups of proteins by the action of di 2 chloroethylsulphone does not reduce the capacity of the proteins to combine with suramin.

7 Suramin does not react with the thiol groups of cysteine or proteins.

The authors are pleased to express their indebtedness to the Medical Research Council for grants which have partly defrayed the cost of this investigation. We are also grateful to Miss H. M. Dewey for help with one of the experiments described in this paper.

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The Phosphorus Compounds of Wheat Starch

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(Received 2 March 1949)

In a previous paper, Lampitt, Fuller & Goldenberg (1941) showed that the insoluble portion separating out when wheat starch retrogrades has a much higher phosphorus content than the soluble fraction, and the presence of a phosphorus rich fraction in wheat starch was postulated.

In the extensive literature on the phosphorus compounds of starches (summarized with many references by Lampitt *et al.* 1941, Lampitt, Fuller & Goldenberg, 1948), the consensus of opinion appears to be that, whereas in potato starch the phosphorus is present as an amylophosphoric ester

in wheat starch it is combined as phosphatide loosely bound to carbohydrate, possibly by polar adsorption. This phosphatide, however, has apparently not been isolated and characterized, its presence being presumed by several authors on not very conclusive evidence. That wheat flour contains a phosphatide was shown by Winterstein & Hiestand (1907) who demonstrated in the gluten residue obtained on washing out starch from a dough the presence of a substance, associated with reducing sugar, which contained choline and gave β glycerophosphoric acid on acid hydrolysis. Nottbohm & Mayer (1934)

found that most of the choline present in wheat flour remains in the starch fraction washed out of a dough, the starch contained no free choline, but 90 % of the choline present was isolated in a form soluble in benzene after the starch had been ground with pumice powder and water (It is to be noted that the phosphatide content calculated from the choline values accounted for only 35 % of the total phosphorus present in the starch) By acid hydrolysis of wheat starch, Posternak (1935) obtained only glycerophosphoric acid and no glucose 6 phosphate Schoch (1942) showed that most of the fatty matter and most of the phosphorus in wheat starch were soluble in 85 % methanol or 80 % dioxan, the soluble fraction having the high phosphorus content of 2.25 % Lehrmann (1930), by extraction of the solution resulting from the acid hydrolysis of defatted wheat starch, obtained 1 % of fatty matter consisting of fatty acids, but the aqueous solution contained no nitrogen and no glycerol Lehrmann, who made no attempt to determine how this fatty acid was combined in the starch, later (Lehrmann, 1945) confirmed Schoch's (1942) results He agreed with the conclusion of the latter, on the meagre evidence adduced, that the major proportion of the phosphorus in wheat was present in the form of loosely bound phosphatide, a small proportion of the phosphorus being combined directly with carbohydrate

From the extensive literature, it appears that phosphorus is combined in the wheat berry in the following forms (a) inorganic phosphate (probably potassium dihydrogen phosphate), (b) phytin (chiefly in bran and germ), (c) phosphatide, (d) nucleic acid (especially in germ), (e) associated with the starch, probably as a starch phosphoric ester, (f) possibly also hydrolysis products of these It is possible that any or all of these types of phosphorus may be present in wheat starch, even if only as contaminants due to incomplete separation of the starch from the other flour constituents It is also possible that decomposition products of these phosphorus compounds may be produced during isolation of a fraction

Preliminary work showed that starch yielded very little soluble phosphorus and nitrogen when extracted by the normal method for phosphatides using ethanol and ether The starch was therefore completely peptized with chloral hydrate as a preliminary step, the method of Meyer, Brentano & Bernfeld (1940) being chosen as it was claimed that degradation and hydrolysis of the starch molecule are thus avoided The peptization was followed by precipitation with acetone, both soluble and insoluble fractions being examined

EXPERIMENTAL

The starch used This was prepared in the usual way by washing out from a dough made from a strong wheat flour

of 70 % extraction The starch was allowed to sediment out from the wash water, washed by decantation several times with water and finally filtered on a Büchner funnel The starch was dried in a current of hot air at a temperature below the gelling temperature, the lumps of dried starch pulverized and passed through a 48 mesh silk sieve Microscopical examination showed that very few swollen and burst grains were present Two samples of starch used contained approximately 8 % moisture, and (in the dry solids) 0.08 % P and 0.11 % N (N/P, 3.0/1), and 0.07 % P and 0.08 % N (N/P, 2.8/1), respectively Starch prepared by the method used in this investigation invariably contains free phosphate In the present case, 14 % of the total P in the starch was extractable by shaking for 1 min with water at room temperature, and this fraction was almost entirely inorganic P This 'adventitious P' has been borne in mind during this study, it is considered that its presence does not affect the main conclusions reached

Methods of analysis Nitrogen was determined by the micro Kjeldahl procedure using Cu as catalyst, phosphorus by the Kuttner & Lichtenstein (1932) method after destruction of organic matter with H_2SO_4 and H_2O_2 (except where otherwise mentioned N and P values are calculated as percentages of the respective total N and P contents of the original starch and N/P ratios are calculated in terms of gram atoms), inorganic P by colorimetric estimation after precipitation with magnesia mixture

Choline was determined where there was sufficient material, using the colorimetric reineckate method of Engel (1942) Two modifications in this method were found to be desirable (1) hydrolysis with baryta for 3 hr instead of 2 hr, (2) washing the reineckate precipitate with 3×2 ml. portions of ice cold water according to the method of Ramsay & Stewart (1941), it was established that under these conditions the solubility of the precipitate was only 6 mg/100 ml so that the loss on washing was negligible When the acetone solution of the precipitate was unsuitable for colorimetric measurement of the colour (e.g. if the colour was too weak or the solution slightly turbid), the amount of choline present was calculated from the N content (micro Kjeldahl) as described by Ramsay & Stewart (1941), it was found that the colorimetric and N methods gave identical results

Glycerol was determined by the periodate procedure of Ramsay & Stewart (1941) To obtain satisfactory results with this method, it was found essential to proceed under rigidly controlled conditions and to observe the following precautions (a) not more than 6 ml. of the baryta hydrolysate of the phosphatide should be taken when the volumes of reagents specified by Ramsay & Stewart are used (b) the time of oxidation should be exactly 5 min, (c) distillation of the formaldehyde produced should continue until the solution is almost dry before water is added prior to the second distillation, (d) the liquid must remain slightly alkaline during distillation to avoid evolution of formic acid (also formed on oxidation) which interferes with the subsequent colorimetric determination of the formaldehyde

Peptization in chloral hydrate

The general procedure was as follows 9 g. portions of starch were each dissolved by heating to 80° with 300 ml 33 % (w/v) aqueous chloral hydrate solution (previously brought to pH 7.0 by addition of a little NaHCO_3) and the whole maintained at 80° for 30 min Microscopical examina

tion showed that the starch grains were then completely dispersed. The starch was recovered as a fine precipitate by blowing the hot solution through a fine jet into 600 ml of dry acetone which was vigorously stirred. The solution was decanted off and the precipitate washed by decantation with 2×200 ml. portions of dry acetone, finally filtered on a Büchner funnel and dried at $95-100^\circ$ in a steam oven. The weight of precipitate was practically equal to that of the original starch taken.

The acetone solution and washings (referred to later as 'acetone filtrate') were combined and concentrated to small bulk (50 ml.) under reduced pressure at a temperature below 60° . The concentrate, which contained chloral hydrate, was treated with 50 ml. water and again concentrated as before, whereby a small part of the chloral was removed. Further treatment to remove chloral hydrate by distillation with water was not carried out owing to the possibility of hydrolysis or other decomposition of P compounds.

In three such experiments the acetone filtrate contained on the average 60% of the original phosphorus and 45% of the nitrogen of the starch, and the N/P ratio was materially lower than that of the starch, indicating that fractionation of the P compounds had been effected.

Fractionation of the phosphorus compounds in the acetone filtrate

Five fractionations were carried through on the acetone filtrates obtained from 18 g portions of the wheat starch using the procedure shown in Fig 1.

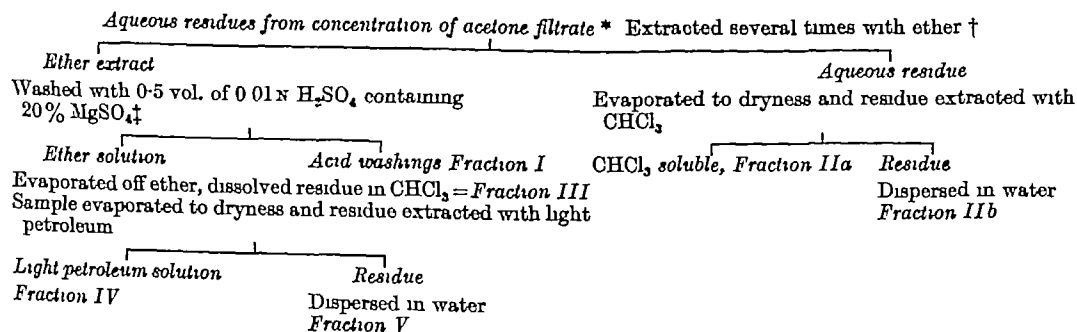


Fig 1 Fractionation of P compounds in acetone filtrate

* All concentrations were effected *in vacuo* at temperatures below 60° , in some cases with N_2 instead of an air leak.
 † As a considerable amount of emulsion formed during the first ether extraction, the mixture was allowed to stand overnight before separation. No difficulty was then experienced in subsequent extractions.
 ‡ This was to remove any non phosphatide N (Kirk, 1938).

Table 1 Fractionation of acetone-soluble phosphorus compounds

	Original starch	Starch ppt	Fractions from acetone filtrate					
			I	IIa	IIb	III	IV	V
Percentage of total P	100	37	0.6	52	2.7	1.0	Trace	Trace
Percentage of total N	100	54	1.5	24	3.8	2.1	1.2	1.7
Ratio N/P	2.6/1	3.7/1	6/1	1.15/1	2.7/1	5.6/1	—	—
Percentage of N in fraction combined as choline	—	—	—	95	—	93	—	nil

Isolation of phosphatide

The acetone filtrate prepared from a total of 112 g of wheat starch was fractionated into the ether soluble portion and the aqueous residue, as described in Fig 1. These two portions were further fractionated by the method outlined in Fig 2, when six fractions were obtained. These were dissolved in suitable solvents and samples taken for analysis. From the results shown in Table 2, it was concluded that Fraction D consists largely of phosphatide, as the N present was mostly in the form of choline, and the ratio of choline N/P was close to 1/1. Further, a positive test for glycerol was obtained when the method of Ramsay & Stewart (1941) was applied to this fraction.

It will be noted that small amounts of choline were present in the other fractions, but in the largest fraction (A) most

of the N was not present as choline. The fraction which appears to be mostly phosphatide (D) was again soluble in CHCl_3 , but insoluble in light petroleum.

Purification of phosphatide

A further quantity of fraction D was isolated by the procedure of Fig 2 from 86 g of wheat starch. The material was purified by solution in CHCl_3 and precipitated with excess of acetone, repeated three times. The product (50 mg) was a white waxy solid (Found: P, 3.85, N, 1.76, choline, 15.64, glycerol, 11.8. Calc. for oleopalmitophosphatide of mol wt 777, P, 4.0, N, 1.8, choline, 15.6, glycerol, 11.8%).

These results indicate that a phosphatide is definitely present in wheat starch, having a composition similar to that of lecithin.

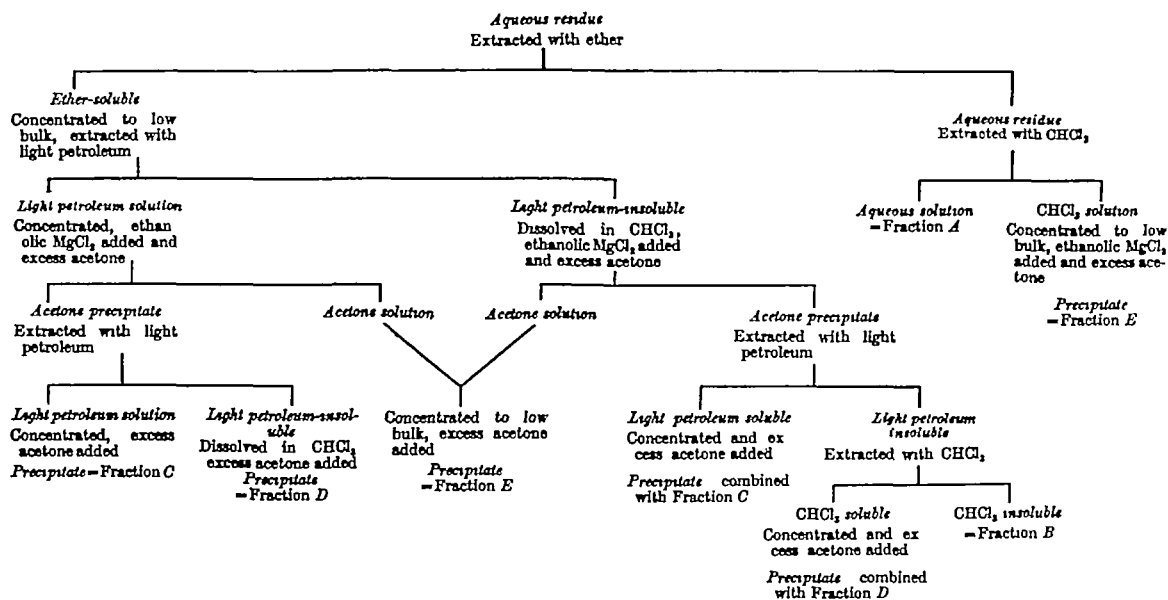


Fig 2 Further fractionation of acetone filtrate. All acetone filtrates combined to form Fraction F. All acetone precipitations made at 0–5°.

Table 2 Analysis of fractions obtained by procedure in Fig 2

Fraction	A	B	C	D	E	F
Wt of fraction (g)	3.96	1.83	0.011	1.118	0.79	The weight of solid matter in this fraction could not be accurately determined owing to the presence of MgCl_2 ; the fraction contained 2.3 mg P and 4.7 mg N of which 0.9 mg was combined as choline, N/P, 4.5/1, choline N/P, 0.9/1
P (%)	0.158	0.433	Trace	3.13	0.132	
N (%)	0.235	0.450	Trace	1.74	0.118	
Choline N (%)	0.031	0.354	—	1.68	0.105	
Ratio N/P	3.0/1	2.4/1	—	1.2/1	2.0/1	
Ratio choline N/P	0.43/1	1.8/1	—	1.2/1	4.5/1	

Table 3 Phosphorus and nitrogen compounds in acetone filtrate

	Acetone precipitate	Acetone filtrate		Total in acetone filtrate (calc as % of weight of original starch)
		Ether soluble	Aqueous residue	
P as % of total P in starch	45	15.8	40*	P, 0.0370
N as % of total N in starch	70	13.5	21	N, 0.0240
Choline N as % of total choline N in starch	—	9.8	16.9	Choline N 0.0186
Ratio N/P	3.9/1	1.8/1	1.3/1	N/P, 1.5/1
Ratio choline N/P	—	1.3/1	1.05/1	Choline N/P 1.1/1

* In another similar fractionation, 5% of the P of the original starch was combined as inorganic phosphate in this aqueous residue.

*The proportion of phosphatide
in wheat starch*

The P compounds in the acetone filtrate from 18 g of wheat starch were separated merely into the ether soluble and aqueous residue fractions in Fig 2, and determinations of N, P and choline were made on these fractions (see Table 3). It will be noted that the ratio of total choline N to total P in the acetone filtrate is 1.1/1, from which it appears reasonable to assume that the bulk of the choline was initially present as phosphatide. From the total choline figure for the acetone filtrate, the calculated phosphatide content is 1.0%, and from the total P content of this filtrate 0.9%. These values, which are obviously maximum values, are comparable with the value of 0.86% calculated from the total P contents of Fractions IIa and III in Table 1.

*Examination of the acetone precipitate (starch residue)
from the chloral hydrate peptization process*

The starch residue obtained by removal of the solvent from the acetone precipitate and drying in the steam oven was soluble in 0.5N HCl, but was precipitated on addition of an equal volume of ethanol. The ethanolic filtrate contained 10–15% of the P of the original starch (the acid soluble P), while approx. 30% remained associated with the precipitate. Similar results were obtained respectively for the extract and the insoluble residue obtained on refluxing the acetone precipitate with 85% methanol for 12 hr. Part of the acid soluble P was combined as inorganic phosphate and part as organic phosphate (glycerophosphate or hexose phosphate), but there is a possibility that some may have been combined as nucleotide or phytin or even as phosphatide liberated by the acid from a complex with carbohydrate. A larger quantity of the acetone precipitate was therefore extracted with 0.5N HCl and the extract fractionated.

The acid soluble phosphorus fraction. The dried acetone precipitated material was moistened with a little alcohol (to avoid the formation of lumps later), 20 times the weight of 0.5N HCl added and the mixture shaken for 1 hr at 15–20°. An equal volume of ethanol was added, and portions of the solution obtained on filtering off the precipitated starch were treated with (a) FeCl₃ after neutralization and (b) excess of Ba(OH)₂, as in the scheme of LePage & Umbreit (1943). The Fe precipitate was materially reduced by solution in acid and reprecipitation by neutralization, so that the presence of phytin was considered to be very doubtful. The Ba precipitate contained a little inorganic P, and an organic P compound containing glycerol but no combined sugar. In all the soluble and insoluble fractions, qualitative tests for guanine, adenine and choline before and after acid hydrolysis were negative, indicating the absence of nucleotide and phosphatide. The acid soluble P is therefore largely glycerophosphate with some inorganic P.

DISCUSSION

The data presented afford proof that phosphatide is present in wheat starch, and therefore the statements in the literature to this effect, based on inconclusive evidence, have been substantiated. The phosphorus present (approx. 0.07%) has been divided into three main fractions: (1) approx. 50–60% of the total phosphorus remains in solution when a chloral hydrate dispersion of the starch is precipitated with excess acetone. This soluble phosphorus is largely present as phosphatide with a little inorganic phosphate. (2) 10% of the total phosphorus is extractable from the acetone precipitate (starch residue) by 0.5N hydrochloric acid, as inorganic phosphate and glycerophosphate. (3) Approx. 30% of the total phosphorus remains associated with the starch residue. The nature of the phosphorus compound in this fraction is being investigated.

SUMMARY

1 Peptization of wheat starch in chloral hydrate solution followed by precipitation with acetone yielded an acetone solution containing 50–60% of the phosphorus in the original starch. From this solution a fraction was separated, soluble in ether and chloroform, in which the N/P ratio was nearly 1/1 and 95% of the N was present as choline. Part of the 'adventitious P' present in the starch was also found in this fraction.

2 By precipitation of the chloroform soluble material, 50 mg of a waxy substance was isolated from 86 g of starch, having a composition agreeing with that of lecithin. The presence of a phosphatide was therefore confirmed.

3 The phosphatide content of wheat starch was approximately 1.0% calculated from the choline content of the acetone solution mentioned above.

4 From the carbohydrate material precipitated by acetone, approximately 10% of the phosphorus in the original starch was extracted by 0.5N hydrochloric acid, 30% remaining associated with the carbohydrate.

5 The acid soluble phosphorus was combined as inorganic phosphate (the residual 'adventitious P') and glycerophosphate.

6 Hexosephosphate and nucleotide were not present, while there was no conclusive evidence for phytic acid.

The authors wish to thank the directors of Messrs J. Lyons and Co. Ltd., in whose Laboratories this investigation was carried out, for permission to publish.

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Studies in Vitamin A

9 THE ROLE OF THE THYROID IN CAROTENE AND VITAMIN A METABOLISM

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(Received 4 March 1949)

Apart from the action of the tocopherols in preventing the oxidation of carotenoids in the intestinal lumen, not much is known of other factors which may control the absorption and metabolism of carotene and vitamin A. Attention has, however, been focused on the thyroid gland in this connexion, especially in regard to the conversion of carotene into vitamin A. Kunde (1926) noted the appearance of vitamin A deficiency in thyroidectomized rabbits fed carotene and a little later von Fellenberg & Gruter (1932) and Fasold & Heidemann (1933) claimed that carotene appeared in the milk of thyroidectomized goats. Abeln (1933) at the same time noted that in guinea pigs the administration of thyroxine adversely affected alike the metabolism of carotene and vitamin A. The clinical aspects of the subject have been reviewed by Drill (1943).

After Abeln's (1933) work there was a long interval before the effect of the thyroid on carotene metabolism was again investigated. Drill & Truant (1947), using the remission of xerophthalmia as criterion of vitamin A production, failed to demonstrate vitamin A formation from carotene in thyroidectomized animals. An objection to this work is that the carotene was administered parenterally and there are serious doubts whether injected carotene is utilized to any great extent (see, e.g., Sexton, Mehl & Deuel, 1946). Canadell & Valdecasas's (1947) experience appears to confirm Drill & Truant's (1947) work, but Remington, Harris & Smith (1942) state that eye symptoms in thyroidectomized rats are cured by the oral administration of carotene.

Di Bella (1940*a, b*) also found carotene effective in such animals but with 'reduced efficiency'.

Johnson & Baumann (1947), using the storage of vitamin A in the liver as the criterion of carotene conversion, found that the same dose of carotene produced less liver vitamin A in thiourea treated animals than in controls and that, perhaps surprisingly, control rats stored less than did rats dosed with desiccated thyroid. Administration of thyroxine and thiourea together produced normal liver storage, this indicated that the action exerted by thiourea was antithyroid and that it did not reduce liver storage of vitamin A by virtue of another (unknown) pharmacological action. Kelley & Day (1948) have confirmed the findings of Johnson & Baumann (1947).

Wiese, Mehl & Deuel (1948) emphasize the importance of allowing for the growth inhibiting power of thiouracil when the effect of the drug on carotene metabolism is assessed by means of biological assays involving the measuring of weight increments. They proved that growth inhibition was due to thiouracil *per se*, and not to its action on carotene metabolism, by demonstrating that a normal response could be elicited in thiouracil treated animals when desiccated thyroid, but not vitamin A, was added to the diet. Wiese *et al* (1948) overcame this difficulty with regard to the bioassay by evaluating the dose of carotene required to produce half the maximum growth obtained in the control and experimental groups. Using this technique they found that β carotene was equally effective in both groups.

These experiments could explain the 'reduced efficiency' of carotene as a source of vitamin A noted by Di Bella (1940*a, b*) in thyroidectomized animals.

The present investigation was planned on the assumption that there was an *a priori* case for implicating the thyroid in the control of the conversion of carotene into vitamin A. Further, none of the investigations just discussed indicates the possible mode of action of the hormone and the antithyroid drugs. It seemed likely that one of three possibilities could explain the adverse action of thiouracil (*a*) that the enzyme converting carotene into vitamin A in the intestinal wall is inhibited, (*b*) that the stability of carotene in the intestinal tract is reduced, and (*c*) that the absorption of carotene from the lumen is inhibited.

These three possibilities have been explored, preliminary notes of part of this work have already appeared (Goodwin, 1948, Cama & Goodwin, 1949).

EXPERIMENTAL

Animals The rats and rabbits used were kept under normal laboratory conditions. Rabbits were maintained on a mixed diet of Lever cubes and green outer leaves of cabbage, whilst rats were maintained on cubes only, water was provided for the rats.

Special diets When on experiment, rats were fed on either Lever cubes, ether extracted Lever cubes, or on the carotene (vitamin A) free diet usually used in this laboratory (Glover, Goodwin & Morton, 1948).

Rabbit experiments Rabbits of approx. equal weight were given a daily oral dose by stomach tube for 3 weeks (excluding Sundays) of either thiouracil or desiccated thyroid supplied by Evans Medical Supplies Ltd. Blood samples were drawn from a marginal ear vein at weekly intervals during and for some time after the administration of the thiouracil and the desiccated thyroid.

The blood samples were centrifuged and the resulting plasmas (about 5 ml. each) were examined for carotene, retinene and vitamin A according to the method in general use in this laboratory (see, e.g. Goodwin & Gregory 1948).

Rat experiments For each experiment rats of the same sex and about equal weights were divided into groups of three. Each group was fed either a known weight of one of the basal diets or the same weight of the same basal diet to which had been added a known amount of either thiouracil, or desiccated thyroid, or a mixture of both. Each experiment was divided into three periods: one of 7 days on the experimental diet, one of 3 days on the experimental diet during which time the rats were dosed orally with either 30 or 50 μg of crystalline β -carotene dissolved in refined arachis oil, and one of 3 days during which the animals were again fed the experimental diet only. During these experimental periods faeces were collected completely each day and stored in the ice chest. The separate faeces were mixed well, and samples taken for analysis. The analyses were always carried out in duplicate and often in triplicate, these always agreed extremely well. The carotene concentration in the faeces was determined according to a method described by Morton & Goodwin in a recent Medical Research Council report (Hume & Krebs, 1949).

Stability experiment Colloidal solutions (30 ml) of β carotene containing about 5 μg /ml were incubated at 37° for 2.5 hr at various values of pH, either alone or with 50 μg of thiouracil dissolved in 5 ml of an appropriate phosphate buffer. At the end of 2.5 hr the pigment was extracted from the aqueous phase with freshly redistilled ether, after removing the ether, the residue was dissolved in a known volume of cyclohexane for spectrophotometric measurements.

RESULTS

Rabbit experiment This experiment was planned on the assumption that if thiouracil inhibited the intestinal 'carotenase' of animals, the plasma of which normally contains no carotene (e.g. rabbits, goats), then it might be expected that the carotene would find its way across the intestinal wall and appear in the systemic blood, this apparently occurred in Fasold & Heidemann's (1933) thyroidectomized goats which produced yellow milk. As there is good evidence to suggest that the conversion of carotene into vitamin A is a two stage process, the first step being the production of vitamin A aldehyde (retinene) which is then quickly reduced to vitamin A (Glover *et al.* 1948), a further possibility was that the conversion of carotene into vitamin A was inhibited at the retinene stage.

Table 1 Plasma vitamin A levels of rabbits dosed with thiouracil and desiccated thyroid

Treatment of animals	Vitamin A in plasma (i.u./100 ml)				
	0	1	2	3	4
Control	139	154	148	151	140
Control	113	124	120	115	120
Thiouracil, 250 mg/day for 18 days	163	167	187	159	127
Thiouracil, 250 mg/day for 18 days	149	187*	—	141	133
Thiouracil, 500 mg/day for 18 days	193*	169	170	148	165
Thiouracil, 500 mg/day for 18 days	175	171	173	149	149
Desiccated thyroid, 200 mg/day for 18 days	104	137	123	117	108
Desiccated thyroid, 200 mg/day for 18 days	—	162	180	169	165
Desiccated thyroid, 200 mg/day for 18 days	133	148	126	123	121

* Doubtful values

The blood of rabbits maintained on a high carotene diet and fed thiouracil were thus examined for carotene and retinene. Because of the facilities available at the time, and of its connexion with the main problem, the effect of thiouracil on the plasma vitamin A levels was also examined. Later the investigation was extended to include the action of

desiccated thyroid as well. The weekly values of the plasma vitamin A levels of rabbits fed varying amounts of thiouracil and desiccated thyroid are recorded in Table 1. It will be seen that neither thiouracil nor desiccated thyroid has any effect on the plasma values.

At no time during this investigation was carotene ever detected. Since carotene was consistently absent in the plasma of the thiouracil-treated animals steps were taken to detect, if possible, traces of retinene. In *cyclohexane* λ_{\max} for retinene is $373 \text{ m}\mu$ (Ball, Goodwin & Morton, 1948). No selective absorption was ever noted in this region in plasma extracts. It was not possible to test for

selectively absorbing compound. Incidentally, the correction procedure gives results for the plasma vitamin A levels in good agreement with those obtained using the antimony trichloride method. For example, the values obtained for two plasmas were 128 and 165 $\text{u}/100 \text{ ml}$, using the correction procedure, and 132 and 159 $\text{u}/100 \text{ ml}$, using the antimony trichloride test.

Stability test. Five experiments in which thiouracil was added to colloidal carotene and incubated for 2.5 hr at pH 7, 4 or 8, failed to reveal any action of the drug on the stability of β carotene. Fig 2 records the results obtained in a typical experiment; there is little quantitative or qualitative difference between the absorption spectrum of β carotene incubated alone, or with added thiouracil. A large

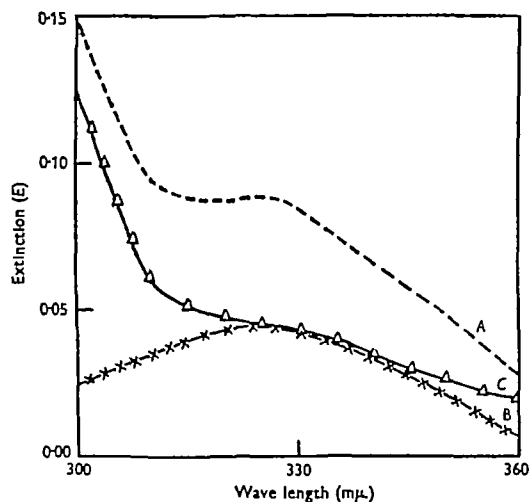


Fig 1 The absorption spectrum of a light petroleum extract of plasma obtained from a rabbit dosed with 100 mg thiouracil for 18 days, ----, direct absorption spectrum (curve A), -x-x-x-, absorption due to vitamin A (curve B), Δ - Δ - Δ -, absorption not due to vitamin A (curve C).

retinene using the antimony trichloride test, in order to identify the retinene band at $664 \text{ m}\mu$ rather than just to measure the absorption at that wave length, the Hilger Nutting visual spectrophotometer has to be used instead of the Beckman photoelectric instrument, this needed more material than was available. In order to see if any other possible vitamin A derivatives absorbing in the region 300 – $360 \text{ m}\mu$ were present the Morton & Stubbs (1946) procedure was applied, a typical result is recorded in Fig 1. After recording the direct ultra violet absorption spectrum of the extract (curve A) the correction procedure was applied to determine the absorption due to vitamin A (curve B), the subtraction curve C (curve A – curve B) records the absorption not due to vitamin A. It will be seen that between 300 and $360 \text{ m}\mu$ the absorption is entirely general and no signs are present of a

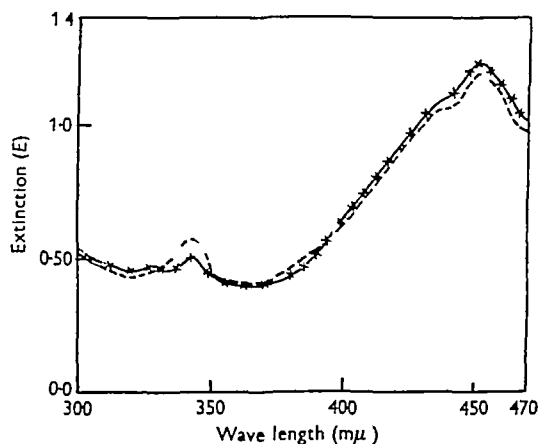


Fig 2 The absorption spectrum of a *cyclohexane* solution of β carotene, ----, after the β carotene had been incubated as a colloidal solution at pH 4 for 2.5 hr at 37° , -x-x-x-, after the pigment had been similarly treated in the presence of thiouracil.

number of similar experiments using desiccated thyroid indicated that this also had little effect on the stability of β carotene. These experiments will be published in detail later for they form part of an investigation into the claim that thyroglobulin converts carotene into vitamin A *in vitro* (Kaplansky & Balaba, 1946).

Absorption experiment. The results of the effect of thiouracil and desiccated thyroid are recorded in Tables 2 and 3. It is plain that even under extreme conditions of absorption (no roughage in the carotene free diet, and a large proportion of roughage in the cubes) thiouracil inhibits and thyroid promotes absorption of carotene from the rat alimentary tract. It is only when very large amounts of carotene are provided in the food that these effects disappear. They are masked because on a large intake a high proportion of the ingested carotene is excreted in any case.

Table 2 *Carotene excretion by rats on various diets with and without the addition of thiouracil (100 mg /day)*

Diet	Duration of experiment (days)	Carotene excreted/day by a group of 3 rats (μg)		Increased excretion in the thiouracil fed groups (%)
		Controls	Thiouracil fed animals	
Ether extracted food cubes	7	4.89	9.32	90.6
Ether extracted food cubes	3	3.34	3.81	11.4
Ether extracted food cubes + 30 μg β carotene/day	3	10.14	15.90	56.8
Carotene free diet*	3	2.43	3.98	63.9
Carotene-free diet + 50 μg β carotene/day	3	7.07	9.53	34.9
Unextracted food cubes	7	60.1	57.3	[- 5.0]
		58.6	54.4	[- 10.7]
Unextracted food cubes + 50 μg β carotene	3	107	112.9	5.0
Mean increased excretion 30.8				

* This was during a period immediately following the administration of β carotene which was still being excreted

Table 3 *Carotene excretion by rats on various diets with and without the addition of desiccated thyroid (100 mg /day)*

Diet	Duration of experiment (days)	Carotene excreted/day by a group of 3 rats (μg)		Decreased excretion of the thyroid fed group (%)
		Controls	Thyroid fed animals	
Ether extracted food cubes	7	4.89	3.04	37.8
Ether extracted food cubes	3	3.34	3.41	[+ 2.0]
Ether extracted food cubes + 30 μg β carotene/day	3	10.14	8.72	14.0
Carotene free diet*	3	2.43	1.05	56.7
Carotene free diet*	3	2.43	1.70†	30.0
Unextracted food cubes	7	60.1	64.9	[+ 8.0]
Unextracted food cubes + 50 μg β carotene/day	3	107.5	106.5†	1.0
Mean decreased excretion 18.5				

* This was during a period immediately following the administration of β carotene which was still being excreted

† In these experiments the dose was increased to 200 mg /day

Table 4 *Carotene excretion by rats on a diet of ether extracted food cubes with and without the addition of both thiouracil and desiccated thyroid (100 mg /day)*

Diet	Duration of experiments (days)	Carotene excretion/day by a group of 3 rats (μg)		Decreased excretion in the group fed thiouracil + thyroid (%)
		Controls	Thiouracil + thyroid fed animals	
Ether extracted cubes	7	4.89	3.19	34.7
Ether extracted cubes	3	3.34	2.95	11.5
Ether extracted cubes + 30 μg β carotene/day	3	10.14	9.83	3.1
Mean decrease				16.4

It was obviously of importance to discover whether the inhibitory effect of thiouracil could be counteracted by the simultaneous feeding of desiccated thyroid. Consequently a similar experiment was carried out in which both desiccated thyroid and thiouracil were included in the basal diet. There is no doubt that the thyroid extract counteracts completely the effect of thiouracil (Table 4) and under the conditions of this experiment also exerted a positive effect in spite of the presence of thiouracil.

DISCUSSION

The results set out in this paper indicate that the thyroid does play a part in the metabolism of carotenoids. It acts, not by stimulating the conversion of carotene into vitamin A, but by controlling the intestinal absorption of carotene. The antithyroid action of thiouracil is also demonstrated by the fact that it inhibits absorption of the pigment and that this effect is counteracted by the simultaneous feeding of desiccated thyroid.

It is now possible to reconcile to some extent the conflicting reports as to the effect of antithyroid drugs on carotene metabolism. Wiese *et al* (1948) used the bioassay to demonstrate the inability of thiouracil to inhibit the growth promoting power of β carotene. It is probable that at dose levels of 1 μ g of β carotene per day the effect of thiouracil would be small enough to go unrecognized by a bioassay with such a high inherent error as that of the vitamin A assay (Gridgeman, 1944) unless a very large number of rats was used.

On the other hand, Johnson & Baumann (1947) used the liver storage of vitamin A as the criterion of efficiency of conversion of carotene into vitamin A and fed their rats at a much higher level (40–60 μ g/day). Obviously, over a period of 15 days, disturbed absorption would considerably reduce the amount of carotene available for conversion and this would readily be detected in a fall in the amount of vitamin A stored in the liver, this is presumably what happened in Johnson & Baumann's (1947) experiments in which liver vitamin A was very much lower in thiourea treated rats than in controls. The discovery of Johnson & Baumann, that rats fed desiccated thyroid stored more vitamin A than did controls, also fits in with the present observations that thyroid stimulates absorption of carotene.

It is interesting to consider briefly the effect of the thyroid on vitamin A metabolism *per se*. The results recorded in Table 1 show that, whatever the effect of the thyroid on the utilization of vitamin A (see, e.g. Baumann & Moore, 1939; Drill, 1943), neither desiccated thyroid nor thiouracil, even in large doses, has a significant effect on the plasma vitamin A levels of rabbits. This indicates that the liver factors controlling the plasma liver equilibrium are unaffected.

It is unnecessary to consider the mass of conflicting evidence available concerning the diagnostic use of plasma vitamin A levels in cases of hyper and hypo thyroidism, for it does seem from our data that they are without value in this respect. Only in pathological conditions in which the liver blood equilibrium is disturbed, can the determination of plasma vitamin A levels be of any help. In other conditions the long term uncontrolled nutritional history of a patient is the most important factor, for, because of the existence of the liver-blood equi-

librium, it is only when liver stores are almost exhausted that any dramatic variations in plasma levels can be expected (Lewis, Bodansky, Falk & McGuire, 1942; Glover, Goodwin & Morton, 1947). A hypothetical example may make the argument clearer: two patients A and B, now living on diets containing only small amounts of vitamin A, develop hypothyroidism at the same time, owing to different previous nutritional histories the liver of A contains 25,000 i.u. and that of B 250,000 i.u. (a variation not beyond experience, cf. Moore, 1937). Owing to the functioning of the liver blood equilibrium the plasma values for both A and B would fall within the normal range. Assuming a vitamin A utilization of 1250 i.u./day (Hume & Krebs, 1949) A's reserves would be exhausted within 20 days and his plasma level would show a sudden drop within about 17 days, this fall could at first sight be correlated with the onset of hypothyroidism. In B's case, on the other hand, 200 days would elapse before such a well marked fall would be evident, this could not normally be correlated with the onset of hypothyroidism.

SUMMARY

1 Feeding large amounts of thiouracil to rabbits on a high carotene diet does not cause the appearance of either carotene or retinene in the blood plasma. This is taken to indicate that thiouracil does not inhibit intestinal 'carotenase'.

2 Neither thiouracil nor desiccated thyroid has any qualitative or quantitative effect on the stability of colloidal solutions of β carotene.

3 Thiouracil inhibits and desiccated thyroid stimulates the absorption of β carotene from the intestinal tract of rats. When administered with thiouracil, desiccated thyroid counteracts the inhibition by the drug.

4 Neither thiouracil nor desiccated thyroid has any effect on the plasma vitamin A levels of rabbits.

5 Reasons are given for suggesting that determination of plasma vitamin A levels are of little or no value in diagnosing thyroid dysfunction.

We are grateful to the Medical Research Council and the Ministry of Food for financial assistance towards the cost of this work and to Prof. R. A. Morton for his interest and advice during the investigation.

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Metabolism of Derivatives of Toluene

3 *o*, *m*- AND *p* XYLENES

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(Received 1 April 1949)

One purpose of the present study of the xylenes was to compare their excretion products in the rabbit with those found after administering the corresponding toluic acids (Bray, Thorpe & Wood, 1949) which are known to be metabolites of the hydrocarbons. There was added interest in that the xylenes are compounds with two similar potential 'centres for conjugation' (Bray, Ryman & Thorpe, 1948) and may be compared with the toluamides (Bray *et al* 1949) which have two dissimilar potential 'centres'.

The literature contains few accounts of investigations of the fate of the xylenes in the animal body. Schultzzen & Naunyn (1867) found that 'xylene' was oxidized in man and the dog to 'toluic acid' and excreted as the glycine conjugate. Curci (1892) stated that all three isomers were oxidized in the dog to the corresponding toluic acids. Filippi (1915) showed that the *o* and *m* isomers were similarly oxidized in the rabbit and excreted unconjugated. More recently, Kuhn & Löw (1939) isolated *p* toluic acid as the main excretion product of *p* xylene in the rabbit. Evidence that hydroxylation may also occur has been put forward. Curci (1892) claimed to have isolated from urine both a xyleneol and a hydroxy-toluic acid after the administration of each of the three isomers to the dog. Yields of 25% or more of the xyleneols were reported, but were based upon weights of obviously impure fractions. The configurations of the hydroxylated metabolites were given, but were based solely upon the colour reactions of fractions or products of doubtful purity. Filippi (1915) found evidence for only a small degree of hydroxylation of *m* xylene in the rabbit and was unable to separate any hydroxylated metabolite of *o* xylene.

In the present investigation the effect of the three xylenes on the excretion of ether soluble acid, reducing substances and ethereal sulphate has been studied. The chief metabolites have been isolated and characterized.

EXPERIMENTAL

Materials and methods

Diet and dosage The rabbits and diet used were as in previous studies in this laboratory (e.g. Bray, Ryman & Thorpe, 1947). The xylenes (L. Light and Co.) were administered with water by stomach tubes. No toxic effects were observed with any of the isomers at doses up to 1.5 ml. The *m* and *p* isomers could be given safely in doses of 2 ml, but the *o* isomer at this dose level occasionally caused the excretion of large amounts of reducing material. This differed from the expected metabolite in that it was not soluble in ether (of ether solubility of the ester glucuronide metabolite of *o* toluic acid (Bray *et al* 1949)). The reducing material may be a manifestation of a toxic reaction, similar observations were made with *o* toluamide (Bray *et al* 1949) and *o* and *m* acetotoluidides (Bray & Thorpe, 1948).

Methods These were for the most part the same as those used in the study of the metabolism of the toluic acids reported previously (Bray *et al* 1949). In some experiments the reducing power of hydrolysed urine was determined using the method described by Bray, Neale & Thorpe (1946b).

RESULTS

Normal excretion of metabolites

The average daily excretion of ether soluble acid by individual rabbits ranged from 683 to 895 mg (calculated as hippuric acid). In the majority of experiments, owing to a long delay in excretion, the percentages of the doses corresponding to the increases in ether soluble acid excretion were calculated from single 'base line' values, i.e. those of the days before administration of the dose. Although the results cannot be so satisfactory as those normally obtained using two or more 'base line' values, they serve to show the general quantitative features of the oxidation of the xylenes to toluic acids.

The average daily output of reducing material by individual rabbits ranged from 147 to 195 mg (calculated as glucuronic acid). The average percentages by

which the individual daily values differed from the weekly average base lines used for calculation of results was $\pm 6\%$

The average daily excretion of ethereal sulphate for individual rabbits ranged from 25 to 39 mg SO_3 . The average percentage by which the individual daily values differed from the weekly averages was $\pm 9\%$ (± 3 mg SO_3)

Metabolism of o xylene

The results are summarized in Table 1. As with o-toluic acid (Bray *et al.* 1949), the ester glucuronide formed is soluble in ether and hence is included in the values for ether soluble acid. The average total recovery according to the above results is therefore 66%. In a few experiments at the highest dose level

Table 1 *Excretion products of o xylene in the rabbit expressed as percentage of dose*

(Ranges given in parentheses)

	Dose (g)	No of expts	Day 1 (%)	Day 2 (%)	Day 3 (%)	Total excretion (%)
Ether soluble acid	0.9	3	42 (40-44)	10 (0-22)	0 (0-1)	52 (40-66)
	1.35	14	51 (29-59)	12 (0-24)	3 (0-11)	65 (47-81)
	1.8	12	37 (17-57)	13 (5-22)	3 (0-8)	54 (40-76)
	Av		45	12	3	60
Ester glucuronide	0.9	2	22 (20-23)	0	0	22 (20-23)
	1.35	3	27 (20-35)	3 (0-5)	0	29 (20-38)
	1.8	9	30 (24-36)	2 (0-5)	0	32 (28-38)
	Av		28	2	0	30
Ethereal sulphate	1.35	1	6	2	0	8
	1.8	4	5 (3-6)	2 (0-3)	0	6 (3-9)
	Av		5	2	0	6

used (not included in the table) the reducing value of the unhydrolysed urine was abnormally high. It was found, however, that only a portion of the reducing material was soluble in ether, suggesting as already stated, that the remainder was probably a response to a toxic compound. The amount of ether soluble reducing material excreted in these cases was of the same order as that normally found and recorded in Table 1.

Evidence was also obtained of the excretion of an ether type glucuronide. The determination of ether glucuronide in the presence of ester glucuronide cannot normally be satisfactorily carried out by our method (Bray *et al.* 1946b). In the present instance, however, the ether solubility of the ester glucuronide permits a separation. o xylene urnes (as collected) were, therefore, continuously extracted with ether for 18 hr to remove ester glucuronide and ether glucuronide was then estimated in the extracted

urine. On the basis of six experiments it was estimated that about 10-15% of the dose was excreted as an ether type glucuronide, about a third of this during the second 24 hr after dosage. If this is added to the values given in Table 1, approximately 80% of the dose can be accounted for.

Metabolism of m xylene

The results obtained are given in Table 2. Most of the dose is excreted as ether soluble acid which is composed of both m toluic and toluric acids, the latter predominating (see p 243) and a small amount of an ether soluble ester glucuronide (presumably m toluylglucuronide). About 4% of the dose is hydroxylated, a finding which would appear to agree with that of Filippi (1915). No ether glucuronide was detected.

Table 2 *Excretion products of m xylene in the rabbit expressed as percentage of dose (1.74 g throughout)*

(Ranges given in parentheses)

	No of expts	Day 1 (%)	Day 2 (%)	Day 3 (%)	Total excretion (%)
Ether soluble acid	13	59 (47-74)	14 (3-24)	8 (0-15)	81 (69-95)
Ester glucuronide	8	2 (0-4)	0	0	2 (0-4)
Ethereal sulphate	4	4 (3-4)	0	0	4 (3-4)

Table 3 *Excretion products of p xylene in the rabbit expressed as percentage of dose (1.7 g throughout)*

(Ranges given in parentheses)

	No of expts	Day 1 (%)	Day 2 (%)	Day 3 (%)	Total excretion (%)
Ether soluble acid	15	63 (50-76)	17 (12-24)	8 (0-16)	88 (63-109)
Ester glucuronide	10	1 (0-2)	0	0	1 (0-2)
Ethereal sulphate	4	0	0	0	0

Metabolism of p xylene

Table 3 summarizes the results obtained. Virtually the whole of the dose which can be accounted for is excreted over 3 days as ether soluble acid, the nature of which is described below. No ether glucuronide was detected.

QUALITATIVE EXPERIMENTS

o Xylene. Continuous ether extraction of o xylene urnes gave a dark brown syrup from which o toluic acid was readily isolated. A typical yield was 1.0 g from total doses of 6 ml (5.3 g) o xylene. Toluene fractionation of the ether soluble material did not yield any o toluic acid, but in one experiment on a larger scale (total dose 18 ml o xylene) evaporation at ordinary temperature of the mother liquors remaining after the removal of o toluic acid gave 50 mg o toluic acid, m.p. 164° (Gleditsch & Moeller (1889) found m.p. 162.5°) (Found N 6.7. Calc for

Table 4 *Metabolism of xylenes, toluamides and toluic acids in the rabbit*

Compound	Percentage excreted as			Total percentage accounted for	Percentage isolated* as		
	Ether soluble acid less ester glucuronide	Ester glucuronide	Ethereal sulphate		Glycine conjugate	Toluic acid	Other oxidized form
<i>o</i> Xylene	30	30	6	80	0.3	15	—
<i>o</i> Toluamide	—	—	0	20	0	0	20
<i>o</i> Toluic acid	4	73	0	77	0	0	0
<i>m</i> Xylene	79	2	4	85	36	2	—
<i>m</i> Toluamide	85	9	0	94	25	6	28
<i>m</i> Toluic acid	67	26	0	93	23	17	0
<i>p</i> Xylene	87	1	0	88	42	2	—
<i>p</i> Toluamide	70	4	0	74	43	3	0.4
<i>p</i> Toluic acid	84	14	0	98	46	3	0

* These values were those isolated from 24 hr urine, so that amounts actually excreted are certainly greater

$C_{10}H_{11}O_3N$ N, 7.25%) As in the study of *o* toluic acid, we were unable to obtain the ester glucuronide in crystalline form, the usual methods of isolation yielding only syrups which gave some *o* toluic acid on hydrolysis. Preliminary attempts to isolate hydroxylation products were unsuccessful. Most of the xylenols and hydroxytoluic acids give little or no coloration with ferric chloride and the other phenol reagents (e.g. Folin & Ciocalteu, Millon, 2,6-dichloroquinonechloroimide) given by most of the fractions isolated are also given in some degree by normal rabbit urine. It is proposed to re-investigate this aspect of the metabolism of the xylenes after completing a study of the metabolism of the xylenols, which is in progress.

m Xylene The ether soluble material obtained by continuous ether extraction of 24 hr urine of rabbits which had each received *m* xylene (1.5 ml, 1.3 g) was fractionated by refluxing with toluene as described for the toluic acids (Bray *et al.* 1949). The amount of *m* toluic acid isolated from urine corresponding to a dose of 15 ml (13 g) *m* xylene was 8.5 g (36% of the dose) and of *m* toluic acid 400 mg (2.4% of the dose). No hydroxylation product has yet been isolated (see last paragraph under *o* xylene above).

p Xylene The yields of *p* toluic and toluic acids obtained from 24 hr *p* xylene urine as described above were 200 mg and 10.0 g, respectively, from 15 ml (13 g) *p* xylene, representing 1.2 and 42% of the dose. While no hydroxylation product has yet been isolated there is evidence that 2,5-xylene-1-ol may be formed. This compound gives a colour with 2,6-dichloroquinonechloroimide which is characteristically different from that given by normal urine extracts (a brighter blue developing more rapidly) and this colour was obtained on testing the ether-soluble material obtained from *p* xylene urine extracted as collected (pH 7.5).

DISCUSSION

Table 4 compares the results of the present investigation with those obtained with the toluic acids and amides (Bray *et al.* 1949).

The chief features of this comparison are the higher percentages of the doses of the toluic acids conjugated with glucuronic acid, the similarity of the glycine conjugation of the members of each group of isomers and the hydroxylation and ethereal sulphate conjugation of *o* and *m* xylenes.

The results provide a basis for assessing the roles of conjugation with glycine and with glucuronic acid in the metabolism of *m* and *p* toluic acids. It would appear that glycine conjugation of these acids takes place readily, even when their concentrations in blood are small, since there is little difference between the amounts of glycine conjugate derived from the acids administered as such or arising from oxidation of the xylenes or hydrolysis of the toluamides. It is reasonable to suppose that these latter processes involve a slowing down of the overall metabolism, so that the blood levels of the toluic acids would be expected to be lower after the administration of the xylenes or toluamides than after administration of the preformed acids themselves. The lag in excretion that occurs with the xylenes (Tables 2 and 3) also suggests a delayed metabolism, though it cannot be stated whether this is due to slow absorption, excretion or metabolism (absorption seems to have been complete in that no xylene or metabolite of xylene was detected in the faeces). This lag in excretion applies only to the glycine conjugates, since we found that the amounts of unconjugated *m* and *p* toluic acids excreted were very small and that there was no increase in glucuronide excretion after the first 24 hr. These facts suggest that glucuronic acid conjugation of *m* and *p* toluic acids may be a 'shock' metabolic process, occurring only when there is a relatively high blood concentration for which the glycine conjugation mechanism alone is temporarily inadequate, e.g. due to insufficient rate of availability of glycine. Under such conditions it would be expected that glucuronide conjugation would be greater with the toluic acids, ingestion of which would presumably cause a higher blood concentration of toluic acid than would ingestion of the xylene which must first be oxidized to toluic acid. In the case of the toluamides,

if it be assumed that hydrolysis of the amide is a preliminary to conjugation, a delayed metabolism analogous to that of the xylenes and smaller conjugation with glucuronic acid would be expected. The effect is less pronounced than with the xylenes since the hydrolysis of the amide is more rapid than oxidation of the xylene, the metabolites of toluamide being completely excreted in the first 24 hr.

Further support for the view that ester glucuronide conjugation may only occur when there is a relatively high blood concentration of an acid which is readily conjugated with glycine is provided by Snapper & Salzmann's (1947) observations that in man sodium benzoate is excreted as both hippuric acid and benzoylglucuronide whilst benzoic acid is conjugated only with glycine. These authors suggest that sodium benzoate is more rapidly absorbed than benzoic acid. A higher level of blood benzoic acid would thus be achieved by ingestion of sodium benzoate and so favour glucuronide conjugation in addition to glycine conjugation. Such an explanation could also account for our previous finding (Bray, Neale & Thorpe, 1946a) that, although benzamide is apparently completely hydrolysed in the rabbit, less glucuronide excretion was observed with benzamide than with benzoic acid. In experiments now in progress we have also observed that both toluene and benzyl alcohol are oxidized to benzoic acid which at small dose levels are conjugated principally with glycine and to only a small extent with glucuronic acid.

The *o* isomers show clearly the inhibiting effect of an ortho substituent upon the glycine conjugation of a benzoic acid (cf Quick, 1932). The effect appears to be more pronounced in the rabbit than in the dog, in which Quick found 13% of *o* toluic acid to be conjugated with glycine and 79% with glucuronic acid. (The glycine conjugation of the *m*- and *p* toluic acids by the dog was of the same order as we found for the rabbit.) Even with *o* xylene, which of the three ortho compounds would be expected to provide the lowest level of toluic acid in the blood, and, therefore, result in glycine rather than glucuronic acid conjugation, glycine is very small.

There was no evidence of the oxidation of both methyl groups in any of the xylenes. With the toluamides also we obtained no indication of the

conversion of both potential 'centres for conjugation' to carboxyl groups, although it was clear that either of the two centres could be modified, e.g. *m* toluamide formed both isophthalamic acid and *m* toluic acid, but no isophthalic acid, free or conjugated, was detected. It would appear from the results at present available that where a molecule possesses two potential 'centres for conjugation' only one of these is modified in each molecule, although either may be modified in different individual molecules. The evidence for hydroxylation of the xylenes shows that hydroxylation may occur even when there are two potential 'centres for conjugation' in the molecule. The absence of hydroxylation of the toluic acids and amides suggests that the hydroxylation of the xylenes takes place before oxidation of the methyl group.

SUMMARY

1 The metabolism of the three xylenes in the rabbit has been studied.

2 All three compounds are mainly oxidized to the corresponding toluic acids (60, 81 and 88%, respectively). The resulting *o* toluic acid is excreted mainly unconjugated and as an ester glucuronide (30% of the dose), but a small amount (0.3% by isolation) is conjugated. The toluic acids from *m* and *p* xylenes are excreted chiefly as glycine conjugates, only small amounts being excreted free or conjugated with glucuronic acid.

3 Evidence of hydroxylation has been obtained in the case of all three isomers. 6 and 4% of doses of *o* and *m* xylenes, respectively, are excreted as ethereal sulphate and 10–15% of *o* xylene is probably excreted as an ether glucuronide. *p* Xylene may give rise to a xylenol.

4 A comparison of the results of this study with those of a previous one of the toluic acids and amides enables the roles of glycine and glucuronic acid conjugation of the toluic acids to be assessed. The conclusions drawn may also apply to other aromatic acids.

We are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this work and to Mr P. B. Wood for his help in some of the confirmatory experiments. The microanalysis was carried out by Drs Weiler and Strauss, Oxford.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 278th Meeting of the Biochemical Society was held in the Biochemistry Department, University College, Dundee, on Friday, 29 July 1949, when the following papers were read

COMMUNICATIONS

Nucleic Acid Metabolism of *Escherichia coli* By MARJORY STEPHENSON (the late) and JENNIFER M MOYLE (introduced by E F GALE) (*Medical Research Council Unit for Chemical Microbiology, Biochemical Laboratory, University of Cambridge*)

A preliminary study of the nucleic acid metabolism in a strain (*H*) of *Esch. coli* has been carried out using washed suspensions of cells which had been grown on a tryptic digest of casein with 0.5% glucose for 22 hr at 37°

The general phosphorus distribution in the cells was investigated, using Fiske & Subbarow's (1925) method for estimating inorganic orthophosphate. Acid insoluble P amounted to 15–16 $\mu\text{g}/\text{mg}$ cells (dry wt), and acid soluble to 1 $\mu\text{g}/\text{mg}$ cells (of which approximately 45% was inorganic and 55% organic P).

On incubating the cell suspension in bicarbonate buffer (pH 7.4) at 37° in the presence of 0.2 M-Na lactate for 1 hr, the P distribution underwent a marked change. The cells lost 50% of their total P (invariably from the acid insoluble fraction only), all of which appeared in the supernatant, 60% of this being organic and 40% inorganic P.

The acid insoluble fraction of the cells was treated according to the method of Schmidt & Thannhauser (1945) to separate the two nucleic acids. Ribonucleic acid (RNA) amounted to 10–12.2 $\mu\text{g P}/\text{mg}$ cells and deoxyribonucleic acid (DRNA) to 2.7–3.2 $\mu\text{g P}/\text{mg}$ cells. This gives a value of 15% ($\pm 5\%$) for the total nucleic acid. After incubation as above for 3 hr the RNA decreased by 60% and the DRNA by 20–33%.

The organic P fraction appearing in the supernatant was fractionated with barium acetate at pH 8.2, and 90% of the total organic P was found in the 'barium soluble alcohol insoluble' fraction. The molar ratio of pentose/P in this fraction was

1.24, and in the 'barium insoluble' fraction 1.17. Both fractions were completely hydrolysed in N HCl at 100° in 10 min.

No degradation of nucleic acids could be demonstrated in the presence of glucose. The action of Na lactate could, however, be duplicated by equivalent concentrations of NaCl and KCl, though MgCl_2 completely inhibited the breakdown of nucleic acids. Greenstein, Carter & Chalkley (1947) have shown that the rate of breakdown of Na salts of nucleic acids by aqueous animal tissue extracts is very greatly increased in the presence of salts, but here Mg is reported to be relatively more effective than Na.

The breakdown of RNA in intact cells was apparently inhibited almost completely by the addition of inorganic phosphate (40 $\mu\text{g P}/\text{mg}$ cells) and ammonium sulphate (10 mg N $\text{NH}_3/100$ mg cells) to the reaction mixture. In this case, a source of energy was required, since replacement of Na lactate by NaCl gave no inhibitory effect.

That cell suspensions previously boiled 30 min underwent no change in nucleic acids in the presence of salts suggested that these changes were enzymatically controlled. Acetone powders prepared from suspensions of crushed cells possessed activity similar to that of intact cells, and extraction of the acetone powders with buffers gave cell free extracts which were active towards RNA (and sometimes towards DRNA). The rate of breakdown of the RNA of boiled suspensions of crushed cells by such extracts was linear for the initial 20 min and was optimal at pH 7.0.

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The Configuration at C-20 of the Urinary Pregnane-3(α) 17(α) 20-triol of Butler and Marrian
By W KLYNE (*Postgraduate Medical School, London, W 12*)

Butler & Marrian (1937, 1938) isolated from the urine of patients with adrenal virilism a pregnane-3(α) 17(α) 20 triol. Subsequently, Mason & Kepler (1945) isolated from similar material a compound which was apparently the same triol. Mixed melting point determinations on the triols and their diacetates, kindly supplied by Prof G F Marrian, F R S, and Dr H L Mason, have shown that the two triols are identical. All the following melting points are corrected. Triol diacetates: Marrian (purified by chromatography), 155–7°, Mason, 157–9°, mixture, 155–8°. Triols: Marrian (by hydrolysis of the diacetate), 247–9°, Mason, 246–9°, mixture, 246–8°.

The configuration of the urinary triol at C 20 has not been discussed previously. It has now been shown by means of the method of molecular rotation differences that the triol is a 20 α hydroxy compound. (All configurations at C 20 in this paper are allotted following the convention of Fieser & Fieser, 1948.)

The optical rotation of the triol quoted by Mason & Kepler ($[M]_D - 13 \pm 6^\circ$) was compared with those calculated for the 20 α and 20 β isomers from the rotations of the analogous compounds in the 3(β) hydroxyallopregnane series (compounds O and J, Steiger & Reichstein, 1938), using the molecular rotation difference tables of Barton & Klyne (1948). The comparison (Table 1A) gave no conclusive answer, since the calculated $[M]_D$ values for the triols differ only by about 15°, it appeared that the urinary triol was probably the 20 α compound.

Table 1 *Molecular rotations*

(Solvents are indicated by formulae in brackets. Values in italics are calculated from the tables of Barton & Klyne, 1948.)

Compound	$[M]_D$	
	<i>allo</i> Pregnane 3(β) 17(α) 20 triol	Pregnane 3(α) 17(α) 20 triol
A Triols		
20 α isomer	-42° (MeOH)	-4°
20 β isomer	-27° (EtOH)	+11°
Urinary triol		-13° (EtOH)
B Triol diacetates		
20 α isomer	-126° (Me ₂ CO)	+23°
20 β isomer	+103° (Me ₂ CO)	+252°
Urinary triol diacetate		+3° (CHCl ₃)

The calculated $[M]_D$ values for the triol diacetates, however, differ by about 230° (Table 1B), and the configuration of the urinary triol at C 20 could thus be allotted with certainty, using the $[M]_D$ value of its diacetate. The rotation of Prof Marrian's triol diacetate was therefore determined

$$[\alpha]_D^{23} + 0.8 \pm 0.8^\circ \quad (c = 1.5 \text{ in chloroform}),$$

$[M]_D + 3 \pm 3^\circ$. This value, when compared with the calculated values for the isomeric diacetates, shows clearly that the urinary triol is

pregnane 3(α) 17(α) 20 α triol

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Iron Metabolism in Hens' Eggs during Development of the Embryo By W N M RAMSAY
(*Department of Biochemistry University of Edinburgh*)

Sendju (1927) determined haemoglobin in 7-, 14 and 19 day chick embryos by the Sahli acid haematin method after washing the pigment out of the vessels. Schonheyder (1938), in a more extensive investigation, used the rough pyridine haemochromogen technique developed for yeast by Anson & Mirsky (1929). McFarlane & Milne (1934) determined Fe in the chick embryo and its liver, but their technique required them to discard the highly vascular yolk sac.

In the present work haem pigment synthesis has

been indirectly estimated in incubated, frozen, homogenized eggs from the difference between total Fe (modified from Ramsay, 1944) and 'non haem' Fe, defined as Fe reacting with 2·2' dipyridyl in the presence of NH₄OH as reducing agent (Hill, 1931, Sherman, Elvehjem & Hart, 1934, Jones, 1948). The total Fe of 101 eggs averaged 1.07 mg/egg (s.d. ± 0.20 mg, distribution approximately normal). In twelve unincubated eggs 98% (range 95–101%) of the total Fe was found to be 'non haem'. The technique as used at present is thus not sensitive

enough to follow the small but definite synthesis of Hb which takes place during the early days of incubation. Haem Fe, however, increases smoothly and steadily from about 0.08 mg/egg (8% of total) at 8 days to 0.45 mg (40% of total) at 14 days and 0.8–1.1 mg (64–75% of total) at 19–21 days. The amount of haem Fe towards the end of incubation appears to depend largely on the total Fe. The figures quoted for haem Fe may be used to calculate Hb values, which seem about 50% greater than those of Schonheyder, and much greater than those of Sendju.

If haem-Fe is compared with embryo weight (figures of Byerly (1932), compiled from over 2000 eggs), it appears that during the middle period of incubation the rate of haem synthesis is relatively greater than that of increase in gross body weight (wet or dry). Thus, at 14 days, the embryo weight is about 35% of its final value, whereas 50% of the final amount of haem has been synthesized. This is doubtless related to the existence and growth of the important extra corporeal circulation used by the embryo chick for both its respiration and its nutrition.

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Acid-soluble Phosphorus in Hens' Eggs during Development of the Embryo By W. N. M. RAMSAY (Department of Biochemistry, University of Edinburgh)

The major changes in P distribution during the incubation of the hen's egg comprise the disappearance of phospholipin and phosphoprotein P and the accumulation of inorganic and (presumably) nucleic acid P (Plummer & Scott, 1908; Masai & Fukutomi, 1923). These changes, which involve at least 80%, and probably much more, of the total P in the egg, can hardly take place without the intermediate formation of P compounds of low molecular weight. It has therefore been thought worth while to examine changes in the organic acid-soluble P during incubation. Some preliminary results are now presented.

Eggs after known periods of incubation were chilled in acetone CO₂, stripped of their shells and homogenized while still frozen. The cold suspensions were extracted with cold trichloroacetic acid, and the P in the extracts was fractionated with barium acetate and ethanol at pH 8.3 (modified from LePage, 1948). The method of Allen (1940) proved suitable for the P determinations.

Inorganic P amounts to about 5.2 mg/egg before incubation, rises slowly after the 4th day to 9.8 mg at 12 days, and then even more steeply to 50–60 mg at 21 days. Total organic acid soluble P starts at about 2.1 mg/egg, rises to a maximum of nearly 11 mg at 16 days (with a sharp increase from 5 to over 8 mg between the 10th and 12th days), and then decreases slightly to 9.5–10 mg at 20–21 days. These figures are broadly similar to those of Masai & Fukutomi.

The three barium ethanol fractions into which the organic acid soluble P may be divided behave as follows:

(i) Ba-insoluble organic P remains fairly constant at 0.7–0.9 mg/egg for 8 days, and then rises steadily to 4.5 mg at 21 days. P hydrolysed in 10 min at 100° in N-HCl does not account, even in chicks on the point of hatching, for more than about 20% of this fraction (cf. Needham, Nowinski, Dixon & Cook, 1937), but our technique is not proof against partial loss of ATP or ADP.

(ii) Ba ethanol insoluble P begins to rise from 0.6 mg/egg at 4 days until it reaches a final value of 4.0 mg at 21 days. No further investigation has yet been made of this fraction.

(iii) Ba ethanol soluble P, which varies over the first 4 days from zero to 1.3 mg/egg, rises to nearly 2 mg at 10 days, and then to over 4 mg at 12 days, thus accounting for the sharp jump in total acid soluble P seen at the same period. After this there is a steady fall until at 21 days the figure is only about 1 mg/egg. Since the quantity of this fraction is not decreased by carrying out the Ba ethanol precipitation at pH 12 instead of pH 8.3, it does not seem to contain much amino-ethyl phosphate (Outhouse, 1936).

All figures quoted are the means of analyses on 4–10 eggs. Variations from egg to egg were found to be considerable, and only particularly well-defined changes are described here.

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Are Phospholipids Transmitted through the Placenta? By G POPJÁK and MARIE LOUISE BEECKMANS (*National Institute for Medical Research, London, N W 3*)

It was shown in a previous investigation (Popják, 1947) with the aid of ^{32}P that all foetal tissues in the rat, rabbit and guinea pig are able to carry out the phosphorylating step of phospholipid synthesis. The results strongly suggested that most, if not all, the foetal phospholipids are obtained by synthesis within the foetus. Moreover, studies with deuterium and ^{14}C labelled acetate (Popják & Beeckmans, 1949 a, b) proved that even fatty acids are synthesized from smaller molecules within the foetus. The possibility, however, that a small amount of phospholipid, preformed in the mother, might pass through the placenta could not be excluded. The experiments to be reported were designed to probe this question.

The following argument was the basis of the experiments. Two hours after the injection of $^{32}\text{PO}_4^{3-}$ to a pregnant rabbit, the specific activity of the phospholipid-P in the maternal liver is at the most 5%, and in the foetal tissues (foetal placenta, liver and carcass) 7–15% of the specific activity of the inorganic P in the corresponding tissues. If after the intravenous injection of labelled phospholipids to pregnant animals the specific activity of the tissue phospholipid-P would exceed the activity of the inorganic P in any of the organs, this could only mean an active uptake of whole phospholipid molecules from the maternal circulation. Even if the specific activity of the phospholipid P in the foetal tissues were higher than 15% of the specific activity of the tissue inorganic P, the result would favour the view that whole phospholipid molecules have passed the placenta.

Three rabbits (18, 23 and 28 days pregnant) were injected intravenously with serum containing phos-

pholipids labelled with ^{32}P . Two hours after the injection the animals were killed and tissue phospholipids and inorganic PO_4^{3-} assayed for ^{32}P .

The results of one experiment are shown in Table 1. Although the foetal placenta took up appreciable amounts of phospholipids from the maternal circulation, it did not transmit whole phospholipid molecules either to the foetal liver or to the rest of the

Table 1 *Specific activity of phospholipid P and inorganic P in maternal liver and in foetus 2 hr after injection of serum containing ^{32}P labelled phospholipids, 28th day pregnancy*

	Inorganic P counts/min /mg (a)	Phospholipid P counts/min /mg (b)	b/a $\times 100$
Maternal liver	184	1009	548
Foetal placenta	1395	2880	207
Foetal liver	823	59	7.2
Foetal carcass*	142	9.4	6.6

* Whole foetus without liver

foetus. The radioactivity found in the foetal liver and carcass phospholipids was no more than expected from the radioactivity present in the inorganic P. The results on the 18th and 23rd days of pregnancy were exactly the same, except that the placentae on these dates seem to metabolize phospholipids even more actively than on the 28th day.

The unilateral 'permeability' of the rabbit's placenta to phospholipids is very similar to what was found previously for cholesterol (Popják, 1946), i.e. that the placenta may take up large amounts of cholesterol from the maternal circulation, but does not pass it on to the foetus.

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In Search of a P-precursor of Phospholipids By G. POPJAK and HELEN MUIR (National Institute for Medical Research, London, N W 3)

Zilversmit, Entenman & Fishler (1943) showed mathematically that in tracer studies, in which a single injection of the tracer is given to animals, the specific activity time curves of a substance and its immediate precursor should have a relationship as shown in Fig 1. The principles laid down by Zilversmit *et al* have been applied in a search for a specific P precursor of liver phospholipids (lecithin and cephalin) among the acid soluble phosphates. Rats were the experimental animals. The fractionation of the acid soluble phosphates was done as described by Kaplan & Greenberg (1944).

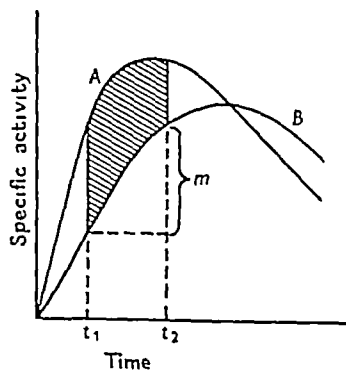


Fig 1 Relationship between specific activity of precursor (A) and product (B)

It was found that after a single subcutaneous injection of $10 \mu\text{c}$ of ^{32}P as Na_2HPO_4 , the only acid soluble P fraction, the specific activity time curve of which fulfilled the criteria for this substance being the specific P precursor of phospholipids, was a Ba, Hg and Pb soluble, but ethanol insoluble, organic phosphoric ester. This fraction was identified as consisting mainly of α and β glycerophosphate. *In*

vivo experiments with both α - and β glycerophosphate, labelled with ^{32}P , failed to substantiate the suggestion that glycerophosphate is a specific phospholipid precursor. The negative results, however, were well explained by the finding that glycerophosphate does not enter liver cells unhydrolysed. *In vitro* experiments with liver slices, in which ^{32}P labelled glycerophosphate, glucose 1- and glucose 6 phosphate, muscle adenylic acid and adenosine triphosphate were used as substrates, similarly gave no evidence that either of these substances might be a specific P donor to phospholipids because of their rapid hydrolysis by tissue slices.

A short interval after the injection of inorganic PO_4 , the specific activities of tissue inorganic P (I), glycerophosphate P (II) and of phospholipid P (III) in descending order are $\text{I} > \text{II} > \text{III}$. It was thought, therefore, that if the reaction between phospholipid and its precursor is a reversible one and if this precursor is really glycerophosphate, then, after the intravenous injection of ^{32}P labelled phospholipids, the specific activities of the P fractions mentioned above should be in the following order $\text{III} > \text{II} > \text{I}$. This argument, of course, should apply only to tissues which take up and metabolize phospholipids. It was found that the rabbit's placenta takes up relatively large amounts of phospholipids from the maternal circulation (see preceding communication), and that 2 hr after the injection of a serum containing ^{32}P labelled phospholipids to a 28 days' pregnant rabbit the specific activity of phospholipid P in the placenta was 2880 c/min/mg, of glycerophosphate P 2090 c/min/mg and of the inorganic P 1395 c/min/mg. It is inferred that the reaction between phospholipid and its precursor is a reversible one and that this precursor is glycerophosphate.

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The Respiration of Rat-Liver Slices in the Presence of some Aliphatic Amines, Hydroxy-amines and Quaternary Ammonium Salts By C LONG (Departments of Physiology and Biological Chemistry, Marischal College, University of Aberdeen)

Eperjessy & Zatureczky (1947) have stated that the addition of very low concentrations (about $2 \times 10^{-5} \text{M}$) of choline chloride, tetramethylammonium iodide, or the hydrochlorides of ethylamine, trimethylamine, ethanolamine or triethanolamine markedly lowers the RQ of liver slices from fasted rats without affecting the rate of O_2 uptake. They interpret this

effect as being caused by a simultaneous stimulation of fat oxidation and inhibition of carbohydrate oxidation by the substances added. Repetition of these experiments has yielded the following results.

(1) Control values for the O_2 uptake by liver slices from fasted rats ($-Q_{\text{O}_2}$ range, 5.1–9.0, av. 6.7) were in agreement with data earlier reported in the

literature and nearly twice as high as the figures given by Eperjessy & Zathureczky ($-Q_{O_2}$ range, calc from their data, 3.1–3.9, av 3.6)

(2) Control values for R_Q , determined simultaneously with (1) above, gave figures in the range 0.33–0.66, av 0.50, also in agreement with earlier literature, but quite different from the range 0.92–1.01, av 0.97, reported by Eperjessy & Zathureczky

(3) Dimethylamine, trimethylamine, triethylamine, ethanolamine, triethanolamine (as hydrochlorides), choline chloride and tetramethylammonium

iodide, when tested at $2 \times 10^{-5} M$, had no effect on the $-Q_{O_2}$ or R_Q values for liver slices from fasted rats. At $2 \times 10^{-2} M$, only choline chloride behaved as a substrate, increasing the $-Q_{O_2}$ and reducing the R_Q

(4) Similar results to those reported under (3) were also obtained with liver slices from well fed rats

(5) When tested directly, the substances examined had no effect on (a) carbohydrate utilization by liver slices from well fed animals or (b) glyconeogenesis in liver slices from fasted animals

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Paper Chromatography of the Vitamin B₁₂ Group of Factors By E. LESTER SMITH and W. F. J. CUTHBERTSON (Research Division, Glaxo Laboratories Ltd., Greenford, Middlesex)

In January 1949 we reported the separation of four active factors from liver extracts and from bacteria by a combination of paper strip chromatography and microbiological assay on a solid medium. Three were characterized as 'slow moving A.P.A.F.', 'crystalline A.P.A.F.' (now known to be identical with vitamin B₁₂) and thymidine. It is now clear (see references) that all five naturally occurring purine and pyrimidine deoxyribosides are microbiologically active, and Winsten & Eigen (1949) showed the presence in liver extracts of six (unidentified) growth factors for *L. leichmanni* 313

ellipticity of the zones of growth on agar, and comparing their maximum diameters with those of standard (undeveloped) rings. The results were independent of the load from 0.003 to 0.8 μg . However, crude products often show imperfect separation of the two zones. In other instances the slow moving factor has clearly split into two.

Some deoxyribosides are difficult to separate chromatographically, especially those of guanine and hypoxanthine, and in some solvents those of adenine and thymine. We have now chromatographic evidence for the presence in extracts from liver and

R_F values in various solvents

Substance	Methyl isobutyl ketone	Methyl ethyl ketone*	Secondary butanol†	<i>n</i> Butanol + 10% acetic acid	<i>n</i> Butanol	Winsten & Eigen <i>n</i> butanol
Slow moving A.P.A.F.	0	0	0.04–0.09	0.10–0.14	0.03–0.08	1 0–0.03
Vitamin B ₁₂	0	Negligible	0.18–0.28	0.25–0.30	0.06–0.13	2 0.03–0.1 3 0.15–0.2
Cytosine deoxyriboside	—	0.09–0.11	—	0.35–0.37	0.28–0.34	—
Guanine	—	0.12–0.14	0.44–0.55	c 0.40	c 0.35	4 0.26–0.36
Hypoxanthine	c 0.01	0.13–0.16	0.44–0.55	0.36–0.46	0.32–0.36	—
Adenine	0.02–0.025	0.28–0.35	0.61–0.69	0.46–0.61	0.51–0.57	5 0.41–0.54
Thymine	0.06–0.065	0.44–0.47	0.71–0.76	0.62–0.73	0.57–0.60	6 0.52–0.67

* 90% saturated with water, developed in cold room. Other solvents saturated with water, developed at room temperature.

† Tank not humidified.

We have extended our work in several directions. Some factors can seriously upset R_F values unless controlled. Volatile (water saturated) solvents tend to evaporate, leaving the paper strip excessively moist, while water evaporates preferentially from other solvents like butanol.

Secondary butanol was suitable for separating vitamin B₁₂ from slow moving A.P.A.F. The portions could be assayed roughly by ignoring the

from *Streptomyces griseus*, of the deoxyribosides of thymine, adenine, cytosine and either hypoxanthine or guanine, the latter correspond with the 'Band 3' we reported previously. The probable identity of five of Winsten & Eigen's six factors is indicated in the table.

The plate assay of liver extracts for vitamin B₁₂ can be made more accurate by removal of the interfering deoxyribosides, this can be done by

measuring microdrops of the test and standard solutions on to a sheet of filter paper and carrying out ascending development with wet *n* butanol for an hour or two, the paper is then cut away beyond the zone of riboflavin used as marker, the resulting

zones of growth have sharp edges and are only slightly elliptical

We wish to thank Dr T Brady, Dr T Jukes and Dr V Koehler for generous gifts of various deoxyribosides

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Irreversibility of the Deamination of Threonine in the Rat By D F ELLIOTT and A NEUBERGER (National Institute for Medical Research, Hampstead, N W 3)

Feeding experiments with amino acids labelled with ¹⁵N in the α position have shown that most of them take part in reversible deamination reactions in the animal body This is true whether the amino acids are essential for growth or not Weissman & Schoenheimer (1941) found, however, that lysine did not exchange its α nitrogen atom for that supplied by other amino acids This is in keeping with the observation of Berg (1936) that D lysine is ineffective for growth purposes The case of threonine, in which the D isomer is known to be ineffective for growth (Rose, 1938), has not hitherto been investigated

Three adult rats, which had been kept for a preliminary period on a low protein diet, were given for three successive days isotopic glycine (150 mg/day/rat) containing 31.5 atom % excess ¹⁵N At the end of the fourth day the animals were killed and the proteins of the internal organs pooled and hydrolysed Serine and threonine were isolated from the hydrolysate by the method recently de-

scribed by one of us (Elliott, 1949), various other amino acids were isolated by well known methods The results of the isotope analyses, given in Table 1,

Table 1 Atom % excess of ¹⁵N in six amino acids isolated from the internal organs of three rats which had been fed glycine containing 31.5 atom % excess of ¹⁵N

Amino acid	Atom % excess ¹⁵ N
Glycine	3.500
Serine	3.300
Threonine	0.000
Arginine monohydrochloride	0.366
Tyrosine	0.107
Lysine monohydrochloride	0.000

clearly show that threonine does not take part in reversible deamination reactions to any detectable extent under the conditions of the experiment We are indebted to Dr R Bentley for the mass spectrometric analyses

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Paper-Partition Chromatography of the Sugars in Urine By MARJORY EASTHAM (introduced by R P COOK) (Department of Biochemistry, University College, Dundee)

The carbohydrates present in samples of urine have been studied by paper partition chromatography (Partidge, 1948) using as partition solvent normal butanol 4, acetic acid 1, water 5

With untreated urines a white mineral trail develops after treatment with ammoniacal AgNO₃, and obscures a number of sugars, but if the urine is treated with basic lead acetate, concentrated and ex-

tracted with methanol, the extract consists mainly of carbohydrates and gives good chromatograms

Normal and diabetic urines and the urine from one case of infective hepatitis and the urine from a patient on caronamide treatment have been studied Variable amounts of glucose are present in all urines A reducing material of R_f value 0.37 is present in large amounts This compound has not

been identified, but the methyl pentose rhamnose gives a similar value and chemical reactions 'Spots' are present at positions suggesting arabinose, xylose and fructose. Uronic acids (including ascorbic acid) are apparently present in all the urines investigated.

A number of reducing 'fast runners' are also found. These have not been identified.

The urine of the patient on caronamide treatment showed a great increase in the amounts of pentoses and uronic acids.

REFERENCE

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The Distribution of Lipoids in the Faeces of Normal and Cholesterol-fed Animals By R O THOMSON (Department of Biochemistry, University College, Dundee)

The distribution of lipoids in the faeces of rats, guinea pigs and rabbits, fed a diet consisting of rat cake (North Eastern Agricultural Co operative Society, Aberdeen), plus olive oil, with and without cholesterol, has been investigated.

The distribution of the main constituents for the control animals is shown in Table 1.

The values for the cholesterol-fed animals are given in Table 2.

Table 2 Cholesterol fed (as percentage total lipid)

Fraction	Rats	Guinea pigs	Rabbits
Unsaponifiable matter	23.5	12.0	19.0
Total sterol	8.5	2.2	4.9
Ester sterol	3.5	0.4	1.9
Unsaponifiable matter not sterol	16.0	9.9	14.1
Total fatty acids	75.7	75.2	61.8
Free acids (a) Calculated	30.1	79.6	49.6
(b) Actual wt	20.3	71.6	21.0
Ester sterol fatty acids	2.6	0.3	1.4
Glyceride acids	52.8	3.3	39.4

Fraction	Rats	Guinea pigs	Rabbits
Unsaponifiable matter	42.6	26.7	47.1
Total sterol	27.4	13.3	18.8
Ester sterol	8.5	1.6	3.7
Unsaponifiable matter not sterol	13.9	13.4	28.3
Total fatty acids	32.5	63.2	39.0
Free acids (a) Calculated	25.8	61.6	28.5
(b) Actual wt	7.8	59.6	6.7
Ester sterol fatty acids	4.2	1.2	2.7
Glyceride acids	20.5	2.4	29.5

The volatile acids present have been estimated and separated by partition chromatography.

Some Observations on the Structures of Certain Fructosans By D J BELL and ANNE PALMER (Biochemical Laboratory, University of Cambridge)

The following preliminary results are reported.

A Inulins Specimens (several times recrystallized from water) were hydrolysed and estimations of D-glucose carried out, using the specific D-glucose oxidase from *Penicillium notatum* (Keilm & Har tree, 1948). Inulin from *Dahlia* was fractionated into rapidly and slowly crystallizing material, inulin from *Taraxacum* and from *Inula* were not fractionated.

The following results were obtained.

Source	D Glucose moles %	Chain length per mole glucose
<i>Dahlia</i> Rapidly crystallized	2.5	38
Slowly crystallized	2.2	43
<i>Taraxacum</i>	2.5	37
<i>Inula</i>	2.3	38
Control (D fructose treated under same hydrolytic conditions)	0.0015	—

The above results of 'chain length' are of the same order as those found by Haworth, Hirst & Percival

(1932) by normal end group assay assuming no glucose to be present. It must be noted that Irvine & Montgomery (1933) showed that hydrolysates of methylated inulin contained some trimethyl D-glucose. It is therefore not clear whether inulin contains glucose as a terminal or an intermediary radical.

B Grass levan Using the partition chromatogram of Bell & Palmer (1949) end group assay results are similar to those obtained by others (vide supra). However, an appreciable amount of dimethylfructose was found among the hydrolysis products. Therefore the levans are either branched polysaccharides or the chains are strongly hydrogen bonded one with another, thus resisting etherification.

Source (grass)	1 3 4 6 Me ₄ fraction (moles)	1 3 4 Me ₃ fraction (moles)	Me ₂ fraction (moles)	'Chain length'
Italian rye	1	12	1	14
Leafy cocksfoot	1	11	1.7	14

Dr A G Ogston of Oxford has determined (by sedimentation and diffusion constants) the molecular weight of the leafy cocksfoot material. This is of the order of 5,200-5,400. Perennial rye grass levan has a similar molecular weight. It is clear that grass levans are not simple chain molecules. We have further shown that bacterial levans are larger in size, since they do not dialyse through cellophane while grass levans are freely diffusible.

C Irisin Using partition and paper chromatography we have shown that methylated irisin contains an appreciable fraction of trimethyl fructoses (missed by previous workers). This consists of about

2 parts of 1 3 4 trimethyl D fructose with 1 part of the (probably) 3 4 6 trimethyl isomer. The results of our experiments are detailed below. (The dimethyl fructose fraction has been shown to be the 3 4 isomer.)

1 3 4 6 Me ₄ fructose (moles)	1 3 4 Me ₃ fructose (moles)	(?) 3 4 6 Me ₃ fructose (moles)	3 4 Me ₂ fructose (moles)	'Chain length'
7	2	1	7	17

Dr Ogston has determined the mean molecular weight of our irisin as 22,500. This indicates that our material contains 8-9 unit chains.

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Observations on the Renal Clearance of some Fructosans By J BEATTIE and A C CORCORAN (introduced by D J BELL) (Bernhard Baron Research Laboratories, Royal College of Surgeons of England, London and Cleveland Clinic, Cleveland, Ohio, U S A)

The renal clearances of levan (from Italian rye grass) and irisin (from iris rhizomes) were determined in unanaesthetized dogs by using either the single dose technique or continuous infusion over

	1st sample (15 min)	End of 1st hour	End of 2nd hour
Levan			
Single dose	1.0	0.35	0.2-0.3
Continuous infusion	0.72	0.48	0.4-0.45
Irisin			
Single dose	0.85	0.62	0.3
Continuous infusion	0.60	0.45	0.4-0.5

a period of 2-3 hr. Simultaneous determinations of creatinine, mannitol and sodium thiosulphate clearances were made. The ratio of fructosan to creatinine clearance declined as shown in the table.

The results suggested that neither of the fructosans was homogeneous. The fall in the absolute fructosan clearance and that relative to creatinine or thiosulphate clearance were independent of plasma level. Histamine in doses sufficient to cause renal vasodilation produced no change in the absolute or relative fructosan clearance.

The Changing Chemical Composition of Chick-Heart Explants Grown *in vitro* By J N DAVIDSON and I LESLIE (Department of Biochemistry, Glasgow University)

By modifying the roller tube technique employed in earlier work on chick heart explants *in vitro* (Davidson, Leslie & Waymouth, 1949), it has become possible to determine the changing amounts of a number of P and N components of the growing cultures. In addition to the measurement of the acid soluble P and lipid P, the ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) fractions are determined by a modified form of the Schmidt & Thannhauser (1945) method, while N is determined by the micro Kjeldahl method (Markham, 1942).

Evidence from other sources is accumulating to show that the DNA content of the nuclei of all somatic cells is constant in amount for each particular

species, excluding, perhaps, those of certain mammalian organs (Vendrely & Vendrely, 1948; Mirsky & Ris, 1949). On this assumption the amounts in DNAP can be taken as directly proportional to the number of cells in the cultures at various stages of growth. The approximately sevenfold increase in DNAP recorded during 7 days' growth implies a similar increase in the number of cells. Most of this increase occurs after the third day of growth.

The chemical composition of the explants during growth changes in a regular fashion, and when expressed as the number of P and N atoms of the various fractions relative to atoms of DNAP, the results can be interpreted as the changes in composition of the cells. The general trend is a decrease

in the amounts of proteom N, acid soluble P, lipid P, and RNAP relative to the DNAP, particularly after 3 or 4 days of growth. The relative proportions of

these components, however, show little change in the later stages of growth, when the DNAP is still increasing appreciably in the cultures as a whole

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Phosphorus Compounds in the Cell Nucleus chemistry Department, Glasgow University)

By J. N. DAVIDSON and W. M. McINDOE (*Bio-*

Isolated cell nuclei from rabbit liver, fowl erythrocytes and calf thymus have been submitted to the fractionation procedures of Schmidt & Thannhauser (1945) and of Schneider (1945) and to a combination of these methods, neither of which is, by itself, altogether satisfactory. The phosphorus content of each fraction was estimated. The results for the nucleic acid fractions have been compared with figures from pentose and deoxypentose estimations by a number of different methods.

Cell nuclei contain acid soluble phosphate esters, phospholipids (relatively abundant in erythrocyte nuclei), deoxyribonucleic acid phosphorus (DNAP) and small amounts of ribonucleic acid phosphorus (RNAP), phosphoprotein phosphorus (PP), protein bound phosphorus insoluble in acid and warm NaOH (IP) and a protein bound phosphate ester (EP).

With the aid of ^{32}P these compounds have been shown to be biologically distinct. When the various

phosphorus fractions are separated from nuclei obtained from animals which have received radiophosphorus, the phospholipid has a low specific activity, the DNAP is found to have a much lower specific activity than the RNAP, the EP has an activity of intermediate value, while IP and PP have very high activities, comparable with those for acid soluble phosphorus. High activity for phosphoprotein phosphorus in whole tissues has already been recorded (Davidson, Gardner, Hutchison, McIndoe, Raymond & Shaw, 1949).

The amount of nucleic acid in 10^{-6} μg /nucleus has been found to be as follows:

	DNA	RNA
Fowl erythrocyte nuclei	2.62	0.09
Rabbit liver nuclei	7.23	0.70

This is in agreement with the results of Vendrely & Vendrely (1948) and of Mirsky & Ris (1949).

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Stercobilin Formation in a Case of Congenital Porphyria and in the Normal A. NEUBERGER and P. H. A. SNEATH

The investigations with glycine labelled with ^{15}N which have been reported (Gray & Neuberger, 1949) have been further extended, especially by carrying out a control experiment on a normal male. The ^{15}N content of the haem in the porphyrin rises to a much greater value than in the normal. The maximum, which is reached after about 12 days, is followed by a marked drop. The later part of the curve appears to be normal, indicating that the majority of the red cells have a life span of about 120 days.

It will be observed from Table 1 that even in the normal the stercobilin excreted during the first few days of the experiment contains a fairly high concentration of ^{15}N . This finding, which necessarily implies that not all the stercobilin can be derived from the breakdown of red cells of a normal life span, might be explained by assuming that a very small number of cells die before they reach the circulation, or have an exceedingly short life span, what might be termed an 'infantile mortality'. It is also possible that a fraction of the stercobilin arises

from the breakdown of haem proteins other than haemoglobin, which may have a rapid turnover rate in the body, or from intermediary porphyrins or their precursors. London, West, Shemin & Rittenberg (1948) have also found a high ^{15}N content of stercobilin in the early period in similar experiments

Table 1 Atom percentage excess ^{15}N of stercobilin in normal male

Days after feeding glycine	Atom % excess ^{15}N
1-4	0.085
5-8	0.161
9-12	0.067
13-16	0.068
20-32	0.018
57-60	0.018

The results obtained from the porphyrin (Table 2) suggest that in this condition a certain proportion of the red cells have an exceedingly short life span. Such an assumption would explain the relatively high ^{15}N content of the haem and of the stercobilin soon after the beginning of the experiment.

There are highly significant amounts of ^{15}N in the stercobilin of the 30th to the 80th days in both cases, but more in the porphyrin. It is not yet clear whether this is due to the breakdown of a very small number of the labelled red cells, or whether it represents stercobilin derived from a second unknown source. During the period when the majority of labelled red cells are being destroyed, the isotope

content of the stercobilin in the case of porphyrin rises. The rise is much less than would be expected from the fall in the haem ^{15}N , and confirms the

Table 2 Atom percentage excess ^{15}N of stercobilin in congenital porphyrin

The ^{15}N content of stercobilin during the first 28 days has already been reported (Gray & Neuberger, 1949)

Days after feeding glycine	Atom % excess ^{15}N
33-37	0.108
38-42	0.080
43-46	0.080
51-56	0.061
57-92	0.064
93-99	0.072
100-105	0.070
106-112	0.061
110-124	0.072
125-131	0.098
132-139	0.091

conclusion that only a small fraction of the stercobilin is derived from the breakdown of red cells of a normal life span. The stercobilin from the case of congenital porphyrin has been shown to be identical with that of normal faeces by elemental analysis, optical rotation and spectral absorption curves.

We wish to thank Dr R. Bentley for determining the isotope concentrations.

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DEMONSTRATIONS

Cup Plate Assay of Vitamin B₁₂ By W. F. J. CUTHBERTSON and JOAN T. LLOYD

Potency Calculator for Use with Logarithmic Dose Response Curves By B. BASIL

The Chemical Changes in Carbohydrates During Growth of the Pea (*Pisum sativum*) By MARJORY EASTHAM (introduced by R. P. Cook) (Department of Biochemistry, University College, Dundee)

Sections of the pea during various stages of growth have been stained using histochemical reagents to demonstrate pectin, starch, cellulose and lignin development.

The soluble sugars have been studied by paper partition chromatography.

Chemical Constituents of the Pea (*Pisum sativum*) By R. P. COOK and MARGARET B. BROWN (Department of Biochemistry, University College, Dundee)

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

The distribution of glycogen in the liver of rabbits By A NYS, X AUBERT and C DE DUVE

Prosthetic groups of the cytochromes present in *Corynebacterium diphtheriae* with especial reference to cytochrome *a*
By W A RAWLINSON and J H HALE

The oxidation of manganese by plant extracts in the presence of hydrogen peroxide By R H KENTEN and
P T G MANN

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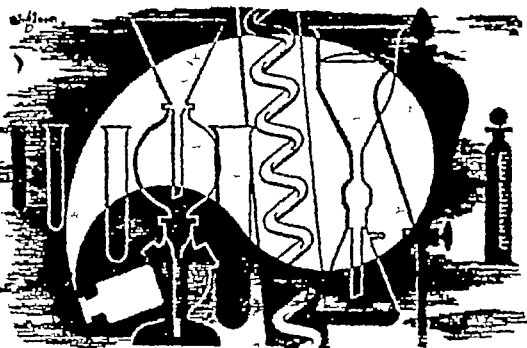
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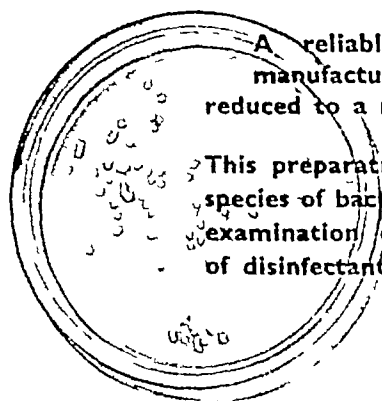
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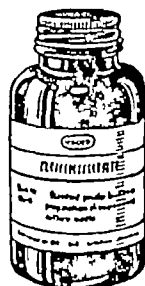
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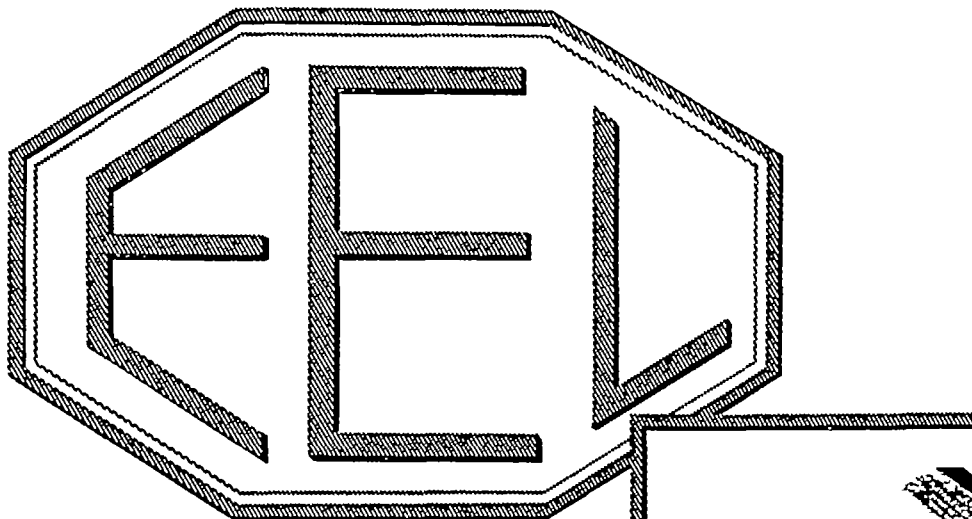
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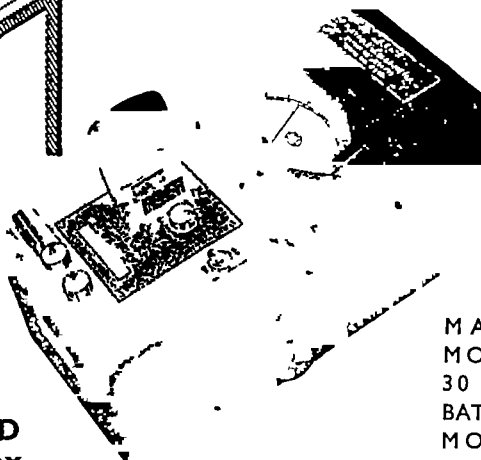
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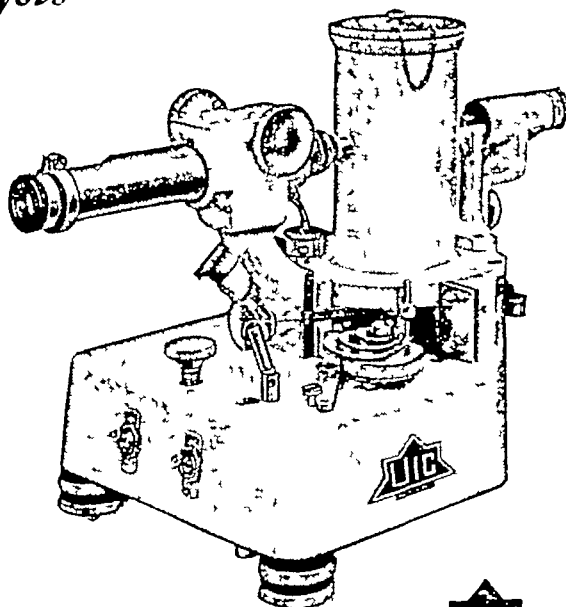
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A Comparative Study of the Succinic Dehydrogenase-Cytochrome System in Heart Muscle and in Kidney

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(Received 22 October 1948)

Most of our knowledge concerning the components of the system of enzymes which catalyses the aerobic oxidation of succinate has been gained from studies of this system in enzyme preparations obtained from heart muscle. Since, in recent years, kidney has been used by many workers as the source of the succinic oxidase system, it seemed desirable to make a comparative study of this enzyme system in kidney and heart muscle. Such a comparison seemed especially necessary in view of the statement of Keilin & Hartree (1940) that kidney (as well as liver and other organs) does not contain a normal cytochrome *b*, which is an essential component of the succinic oxidase system in heart muscle.

This paper describes a study of the succinic oxidase system in the two tissues, using both manometric and spectroscopic methods. A preliminary account of some of these findings has appeared elsewhere (Slater, 1948).

METHODS

Enzyme preparations

Heart-muscle preparation This was prepared from horse heart according to the method of Keilin & Hartree (1947). Minced heart (330 g) was thoroughly washed by stirring with about 5 l tap water for about 15 min. The mince was collected on muslin, squeezed hard to remove water, and this process repeated about 8 times until the wash liquor was colourless. The washed muscle was then ground in a mechanical mortar with 100 g sand (acid washed) and 500 ml. 0.02M phosphate buffer, pH 7.3, for 2 hr. The thick suspension was diluted with 200 ml. 0.02M phosphate buffer and centrifuged for 20 min. at 2000 rev./min. The supernatant cloudy solution was cooled to 0–5° and brought to pH 5.7 with N acetic acid. The precipitate was immediately collected by centrifuging in the cold at 2000 rev./min. for 15 min., the supernatant discarded, and the residue suspended in an equal volume of 0.1M phosphate buffer, pH 7.3. The pH of this preparation was 7.1.

The variation in the activity of different preparations was quite small. The Q_{O_2} of the succinic oxidase system for twenty-one preparations varied between 307 and 740, but was usually between 550 and 700, when fresh heart was used.

Keilin & Hartree (1938) introduced the step involving precipitation with acid for two reasons, viz. (1) to concentrate the preparation in order to make it more suitable for spectroscopic purposes and (2) to remove the last traces of soluble substances such as cozymase, which inhibits the oxidation of succinate by heart muscle preparation (Keilin

& Hartree, 1940), and myoglobin, which interferes with the spectroscopic observations of the cytochromes. In the present study it was found that the precipitation with acid increased the enzymic activity of the original phosphate extract by 50%, probably due to the removal of proteins which are soluble at pH 5.7, as well as to removal of inhibitors. Activities about 20% higher than those obtained by the Keilin & Hartree (1947) method were obtained by centrifuging the neutral phosphate extract at high speed (12,000 rev./min.), instead of acidifying. The activities of the succinic dehydrogenase, the cytochrome oxidase and of the complete succinic oxidase system were all increased to about the same degree. Probably this difference between the activities obtained by the two methods is due to protein which is soluble at pH 7.3, but precipitated at pH 5.7. The amount of this protein will depend, to a certain extent, on the thoroughness of the preliminary washing of the heart-muscle mince.

The succinic oxidase system is distinctly unstable at pH 5.7 at room temperature, and it is important to carry out the precipitation and subsequent centrifugation at below 5°. Since the succinic oxidase system is much more unstable to acid than the succinic dehydrogenase or the cytochrome oxidase (Keilin & Hartree, 1940), the finding that the relative activities of the succinic oxidase system in the two preparations, obtained by high speed centrifugation and acidification respectively, are approximately the same as the relative activities of succinic dehydrogenase and cytochrome oxidase shows that acidification at low temperature does not cause any inactivation of the succinic oxidase system. It was found that the heart-muscle preparation could be precipitated a second time at pH 5.7 at 5°, washed with cold water and resuspended in phosphate buffer without loss of activity, but a further precipitation caused a considerable loss of activity. This is probably due to a physical effect on the macromolecular particles, since the inactivation can be reversed by treatment with various protein preparations (Stern & Melnick, 1939; Keilin & Hartree, 1949). Precipitation at pH 4.6 at room temperature, as used by some authors (e.g. Ogston & Green, 1935), is certain to lead to considerable inactivation.

Kidney preparation Pieces of horse kidney cortex about $1 \times 1 \times \frac{1}{2}$ in. were treated in a Waring blender with 0.02M phosphate buffer for about 5 min. The mixture was centrifuged for 20 min. at 2000 rev./min., and the cloudy red supernatant centrifuged at 12,000 rev./min. for 30 min. in a Servall angle centrifuge. The supernatant was discarded and the residue washed twice with water, centrifuging for 30 min. at 12,000 rev./min. after each washing. The residue was suspended in an equal volume of 0.1M phosphate buffer pH 7.3.

Both the heart-muscle and kidney preparations used in the present investigation contained a considerable amount

of fat, viz 30% in the heart muscle preparation (cf Stern, 1939) and 20% in the kidney preparation

Cytochrome c The cytochrome *c*, which was prepared by the method of Keilin & Hartree (1945), contained 0.34% Fe. The concentration of cytochrome *c* in the solution was determined spectrophotometrically, using a Hilger Nutting spectrophotometer

Measurement of enzyme activities

All measurements of enzyme activities were made in Barcroft differential manometers at 37–39°. Phosphate buffer, pH 7.3, was used in all methods. All activities are expressed as Q_{O_2} (μ l O_2 /mg fat-free dry wt/hr)

Succinic oxidase system The activity of the complete succinic oxidase system was measured in the presence of excess cytochrome *c*. To 0.2 ml of a heart-muscle preparation, diluted fivefold with 0.18M phosphate buffer, were added 2.7 ml 0.18M phosphate buffer and 0.2 ml 1.1% cytochrome *c*, after temperature equilibration, 0.2 ml 0.4M Na succinate was added by dislodging a dangling tube. The equilibration was carried out for not longer than 20 min and at a low rate of shaking, since rapid shaking for long periods caused considerable inactivation of the succinic oxidase system (20% after 1 hr) and of cytochrome oxidase, with little effect on the succinic dehydrogenase. The activity of the kidney preparation was measured in the same way, except that 1.7 ml of the phosphate buffer and 1.0 ml. water were used. Final concentrations were phosphate, 0.15M in the case of the heart-muscle preparation and 0.10M in the case of the kidney preparation, succinate, 0.024M, cytochrome *c*, 4×10^{-5} M. The O_2 uptake between 5 and 15 min after adding the succinate was used as the measure of the activity of the succinic oxidase system. This rate is about 5% less than the initial rate (obtained by extrapolation).

Succinic dehydrogenase To 0.2 ml of an enzyme preparation, diluted fivefold with 0.18M phosphate buffer, were added 2.3 ml (heart muscle) or 1.7 ml (kidney) 0.18M phosphate buffer, 0.3 ml. 0.01M methylene blue and 0.3 ml. 0.1M KCN (neutralized). In the case of the kidney preparation, 0.6 ml water was also added. After temperature equilibration, 0.2 ml 0.4M Na succinate was added by dislodging a dangling tube. Final concentrations were phosphate, 0.15M (heart muscle) or 0.10M (kidney), succinate, 0.024M, methylene blue, 0.0009M, cyanide, 0.009M. The O_2 uptake between 5 and 15 min, or between 5 and 25 min., after the addition of succinate was used as the measure of succinic dehydrogenase activity (the rate of O_2 uptake was practically constant for 15 min after the addition of succinate).

Cytochrome oxidase was determined by the method described in another paper (Slater, 1949a)

Determination of concentrations of haematin compounds

Total protohaematin The total protohaematin content was determined by matching, with a low dispersion micro spectroscope, the intensity of the 548–560 $m\mu$. band obtained by adding pyridine and reducing agent with that of the 551–560 $m\mu$. band of pyridine protohaemochromogen obtained in the same way from pure protohaemin. When pyridine (0.25 vol.) and $Na_2S_2O_4$ were added to the heart-muscle preparation it turned pink in colour and the bands of

cytochrome *c* (548–552 $m\mu$.) and cytochrome *b* (562–566 $m\mu$.) were replaced by a broad band at 548–560 $m\mu$. (cf Keilin, 1926). It was found that the addition of pyridine and $Na_2S_2O_4$ to cytochrome *c* did not alter its absorption spectrum (This may be because cytochrome *c*, which is unusually stable, does not form a pyridine haemochromogen under these conditions. In any case, however, the position and probably the intensity of the band would not be altered since Hill & Keilin (1930) found that the haematin of cytochrome *c* gave a pyridine haemochromogen with bands in the same position as cytochrome *c*.) The short wave length margin of the broad band is therefore derived from cytochrome *c*. The longer wave length portion of this band is due to pyridine protohaemochromogen, derived from protohaematin compounds present in the heart-muscle preparation. A mixture of pure cytochrome *c* and pyridine haemochromogen from pure haemin in the same concentrations as found in heart-muscle preparation gave a band at 548–560 $m\mu$. indistinguishable from that given by heart muscle. The value obtained for the total protohaematin content is probably slightly high, since the cytochrome *c* present in the heart-muscle preparation will show some absorption in the middle of the 551–560 $m\mu$. band, but the error is quite small especially in the case of the kidney preparation, since the total protohaematin content of both preparations is much higher than that of cytochrome *c*.

Cytochrome c The cytochrome *c* content was determined by direct comparison of the intensity of the 548–552 $m\mu$. band obtained by adding $Na_2S_2O_4$ to heart-muscle preparation with the band obtained from pure cytochrome *c*, standardized spectrophotometrically. This value also may be a little high, owing to the probability of absorption of cytochrome *b* at this wave length. However, it appears that this error is not very serious, since treatment of the preparation with ascorbic acid under anaerobic conditions, which causes the appearance of the *c* band only, gave essentially the same results.

Cytochrome b The figures for the cytochrome *b* content are much less certain than the other values, since pure cytochrome *b* is not available for comparison. The figure for the heart-muscle preparation was calculated in the following way. The intensity of the *b* band was considered to be about 20% less than that of the *c* band, it was assumed* that the absorption coefficient of cytochrome *b* is the same as that of cytochrome *c*, since both are haemochromogens of very similar haems. The cytochrome *b* content of the heart-muscle preparation was therefore 80% of the cytochrome *c* content. In kidney preparation the two bands were of about equal intensity.

Cytochrome a + a_3 content The relative cytochrome *a* + a_3 content was determined by comparing the intensities of the band at 603–607 $m\mu$, this is mainly due to cytochrome *a*, but the relative concentration of *a* and a_3 appears to be constant (Keilin & Hartree, 1939).

Weight of the fat free dried enzyme preparation

Enzyme preparation (1 ml) was diluted with 5 ml water in a weighed centrifuge tube, and 1 ml 20% trichloroacetic acid added. The flocculent precipitate was

* The validity of this assumption is supported by the finding of Bach, Dixon & Zervas (1946) that the absorption coefficient of the α band of cytochrome *b* is of the same order as that of cytochrome *c*.

collected by centrifugation, the supernatant siphoned off and the residue washed by centrifugation, once with 5 ml 50% ethanol and once with 5 ml. 96% ethanol. The residue was dried to constant weight at 100°

RESULTS

Examination of the methods of measuring the activities of succinic dehydrogenase and of the succinic oxidase system

Substrate concentration The effect of substrate concentration on the activity of the succinic oxidase system and of succinic dehydrogenase in the heart muscle preparation is shown in Fig 1. High succinate concentrations cause a definite inhibition of the complete system but not of succinic dehydrogenase. This suggests that the inhibitory effect of high succinate concentrations on the succinic oxidase system previously reported by several investigators is not due to a specific action of succinate, but is a salt effect. Other salts, for example phosphate buffer, have the same action on the complete system, with little effect on the succinic dehydrogenase

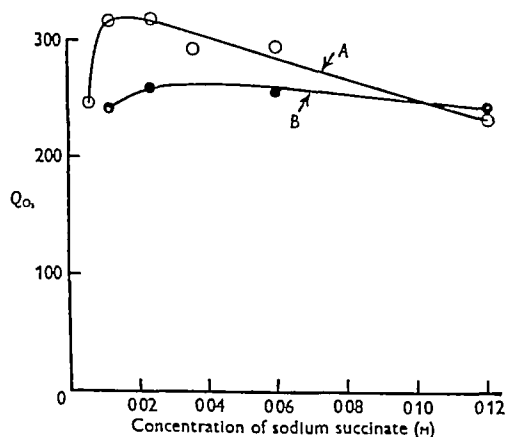


Fig 1 The effect of concentration of sodium succinate on the activity of the succinic oxidase system (curve A) and of succinic dehydrogenase (curve B) in heart-muscle preparation (0.04 ml). Curve A, phosphate, 0.15M, cytochrome c, 4×10^{-5} M. Curve B, phosphate, 0.15M, methylene blue, 0.0009M, cyanide, 0.009M. (Different enzyme preparations were used for the measurements of the succinic oxidase system and of succinic dehydrogenase.)

Concentration of methylene blue Methylene blue is a very sluggish carrier compared with the cytochrome system. The activity of the succinic dehydrogenase was increased by increasing the methylene blue concentration throughout the range shown in Fig 2. However, it was decided to limit

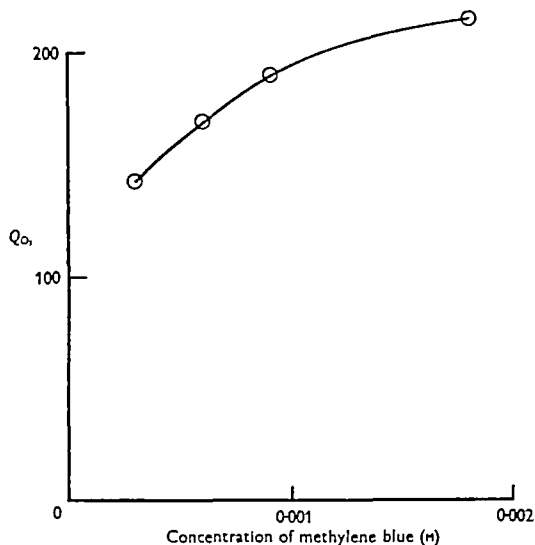


Fig 2 The effect of concentration of methylene blue on the activity of succinic dehydrogenase in heart-muscle preparation, succinate, 0.024M, cyanide, 0.009M, phosphate, 0.15M, 0.04 ml. heart-muscle preparation.

the methylene blue concentration to 0.0009M, even though this concentration was far below the optimum, to avoid the toxic action of methylene blue on the enzyme. The presence of 0.01M cyanide, added to prevent oxidation through the cytochrome system, inhibits the rate of reduction of methylene blue by succinate. For these reasons the absolute values of the succinic dehydrogenase activity determined by the method described have no significance and cannot be compared with the activity of the complete system. This is the explanation of the apparent anomaly that the activity of the complete system in a carefully prepared heart muscle preparation is always greater than that of succinic dehydrogenase, which is an essential constituent of the complete system. The method described is, however, suitable for comparing succinic dehydrogenase activities, if measurements are always carried out in the same way.

(Keilin & Hartree, 1949, Slater, 1949b) The optimal succinate concentration is about 0.02M for both succinic dehydrogenase and the complete system. This is a little lower than the optimum found by Schneider & Potter (1943), using a different type of preparation (kidney homogenate). The finding of Elliott & Greg (1938) that the optimal succinate concentration is less for the reaction with methylene blue than with oxygen is not supported by Fig 1.

Cytochrome c concentration The effect of the concentration of added cytochrome c on the activity of the complete succinic oxidase system in the heart muscle and kidney preparations is shown in Fig 3. With both preparations, 4×10^{-5} M added cytochrome c was sufficient for practically optimal activity.

Enzyme concentration The effect of enzyme concentration on the succinic oxidase and succinic dehydrogenase activities is shown in Fig 4 Under the conditions of the experiments, a straight line, which

(Slater, 1949b) The concentrations of phosphate buffer given in the above methods are approximately optimal for the enzyme concerned

Cytochrome system in kidney preparation

Kidney cannot be washed free from haemoglobin in the same way as heart muscle, since minced kidney is readily dispersed merely by washing in water Accordingly, Keilin & Hartree (1940) used such a dispersion of kidney as the starting material for their preparation, and removed the haemoglobin and other soluble substances by precipitating at pH 5.5 It should be noted that, in this case, the acid precipitation was used to remove all the soluble substances which could be removed in this manner, in the case of the heart muscle, this treatment is used only to remove the last traces of soluble substances which survive the exhaustive preliminary washing of the minced heart muscle As suitable apparatus was available, it was decided to use a different method from that used by Keilin & Hartree (1940) for preparing the enzyme solution The kidney was dispersed in a Waring blender, and the resultant strongly coloured dispersion was freed from soluble substances by centrifugation at high speed and washing the residue with water The method is described in detail on p 1

The preparation showed a very faint band at $580\text{ m}\mu$, probably due to traces of residual haemoglobin On adding succinate, a spectrum essentially the same as that obtained with heart muscle was observed, but with the following differences (1) all bands, but particularly the *b* and *c*, were considerably weaker (see p 5), (2) the *b* band was at $560\text{ m}\mu$, compared with $564\text{ m}\mu$ in the case of the heart-muscle preparation As is the case with heart muscle preparation, the bands disappeared on shaking with air, but reappeared much more slowly than in heart muscle when the shaking was stopped This shows that the succinic dehydrogenase activity relative to that of cytochrome oxidase was considerably lower in the kidney preparation, a conclusion which was confirmed by manometric experiments (see p 5)

When $\text{Na}_2\text{S}_2\text{O}_4$ was used instead of succinate, the *a* band was the same as before, but a strong shading appeared between the *b* and *c* bands, giving the appearance of a single diffuse band with two maxima Thus the kidney preparation contains a substance which gives with $\text{Na}_2\text{S}_2\text{O}_4$, but not with succinate, a band at about $550\text{--}560\text{ m}\mu$, this may be denatured protein haemochromogen At the temperature of liquid air, a kidney preparation diluted fivefold and treated with $\text{Na}_2\text{S}_2\text{O}_4$ showed two absorption bands, a strong band at $555\text{--}559\text{ m}\mu$ and a weaker band at $547\text{--}549\text{ m}\mu$ It seems that at the temperature of liquid air the band due to cytochrome *c* shifts towards the blue end of the spectrum, while the $550\text{--}560\text{ m}\mu$ band is narrowed and intensified and

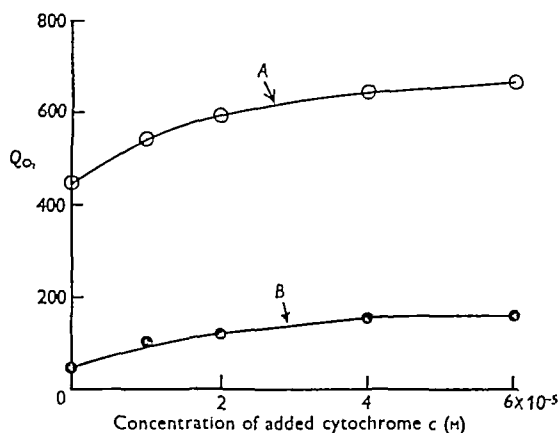


Fig 3 The effect of concentration of added cytochrome *c* on the activity of the succinic oxidase system in heart-muscle preparation (curve *A*) and kidney preparation (curve *B*), succinate, 0.024M , phosphate, 0.15M (heart muscle) and 0.10M (kidney), 0.04 ml enzyme preparation

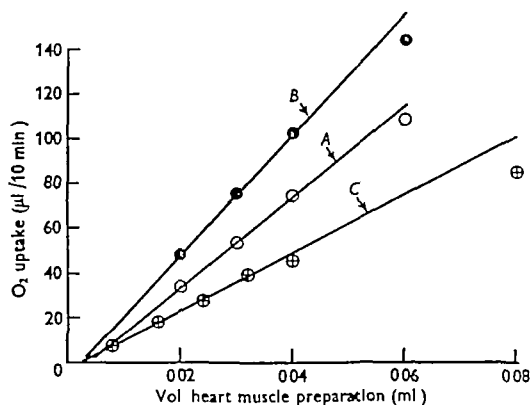


Fig 4 The effect of concentration of heart-muscle preparation on the rate of oxidation of succinate in phosphate buffer (0.15M) alone (curve *A*), in the presence of $4 \times 10^{-5}\text{M}$ added cytochrome *c* (curve *B*), and in the presence of potassium cyanide (0.009M) and 0.0009M methylene blue (curve *C*) Succinate (in all cases), 0.024M

did not pass through the origin, was obtained up to a concentration of 0.04 ml heart muscle preparation per flask (total fluid volume = 3.3 ml) There was a slight falling away from this straight line at higher concentrations

Phosphate concentration The concentration of phosphate buffer has a marked effect on the activities of the succinic oxidase system (Keilin & Hartree, 1949, Slater, 1949b) and of the cytochrome oxidase (Quinlan-Watson & Dewey, 1948, Slater, 1949a), but has little influence on the succinic dehydrogenase

fuses with that of cytochrome *b*, which also moves towards the short wave length

Pyridine and $\text{Na}_2\text{S}_2\text{O}_4$ gave a spectrum essentially the same as that obtained with heart muscle preparation, viz an intense band at 548–560 $\text{m}\mu$ and a weaker band at 580–590 $\text{m}\mu$. The band at 548–560 $\text{m}\mu$ was as intense as with heart muscle, while the 580–590 $\text{m}\mu$ band was much weaker. At the temperature of liquid air, the former band was intensified and sharpened (550–554 $\text{m}\mu$).

Cytochromes *b* and *c* can be readily differentiated by making use of the following properties (1) quinol, *p* phenylenediamine and ascorbic acid reduce cytochromes *c* and *a* only, not cytochrome *b* (a very faint *b* band appears with ascorbic acid, but only after standing for about 30 min), (2) when succinate and phenylurethane are added to the enzyme preparation and the mixture shaken with air, the *b* band alone is visible (cf Keilin, 1925, Keilin & Hartree, 1940). The heart-muscle and kidney preparations behaved in the same way, as regards these reactions. Thus, kidney contains both cytochromes *b* and *c* which appear to have essentially the same properties as in heart muscle. The only qualitative difference is that the cytochrome *b* band occupies a slightly different position.

Keilin & Hartree (1940) found that, in kidney preparations, the usual cytochrome *b* and *c* bands were replaced by a single wider band (with centre at 555 $\text{m}\mu$), which was considered to be the same as that of cytochrome *b*₁ found in certain micro organisms. The present work shows, however, that these observations of Keilin & Hartree (1940) can be explained by the presence of a compound reducible by $\text{Na}_2\text{S}_2\text{O}_4$ which masks the bands of cytochromes *b* and *c*, since these authors used $\text{Na}_2\text{S}_2\text{O}_4$, not sodium succinate, as the reducing agent. If, as is probable, this compound is denatured protein haemochromogen, it would be expected that Keilin & Hartree's (1940) preparation, obtained by acid precipitation, would contain more of this compound than the preparation used in the present investigation, consequently, the two maxima which were observed even with $\text{Na}_2\text{S}_2\text{O}_4$ at room temperature in the present study might be completely masked and only one band be visible. The spectrum obtained with $\text{Na}_2\text{S}_2\text{O}_4$ at the temperature of liquid air was approximately the same as that described by Keilin & Hartree (1940), except that the latter authors give the position of the weaker band as 551 $\text{m}\mu$, compared with 548 $\text{m}\mu$ obtained in the present study. This difference could be explained by the relatively greater intensity of the stronger band in the Keilin & Hartree (1940) preparation compared with that in the preparation used in the present study, since the position of a weak band can be changed by a strong band in the neighbourhood. This weakens Keilin & Hartree's argument that, since at the temperature of liquid air the band

of pure cytochrome *c* shifts to 547 $\text{m}\mu$, the kidney preparation contains no cytochrome *c*.

Comparison of the succinic dehydrogenase cytochrome system in heart and kidney preparations

In Table 1 are compared the activities in heart-muscle and kidney preparations of the complete succinic oxidase system and of its component parts, the succinic dehydrogenase and cytochrome oxidase,

Table 1 *Relative enzyme activities and concentrations of haematin compounds in heart muscle and kidney preparations*

(All activities are based on fat-free dry weight. The methods of determining succinic oxidase, succinic dehydrogenase and cytochrome oxidase activities and the amounts of cytochrome *c*, cytochrome *b*, cytochrome *a* + *a*₃, and total protohaematin compounds are described under 'Methods'. The amount of catalase was determined by comparing the rate of evolution of O_2 when H_2O_2 was added to a suitably diluted heart-muscle or kidney preparation with that obtained, under the same conditions, by adding H_2O_2 to the same enzyme preparation to which a small amount of a solution of pure catalase was added. The concentration of catalase in the latter solution was determined by comparing the intensity of the pyridine haemochromogen band with that obtained from crystalline haemin.)

	Heart muscle	Kidney	Ratio, heart-muscle/kidney
Succinic oxidase (Q_{O_2})	625	200	3.1
Succinic dehydrogenase (Q_{O_2})	240	67	3.6
Cytochrome oxidase (Q_{O_2})	3200	1380	2.3
Protohaematin ($\mu\text{mol/g}$)	1.8	1.8	1.0
Cytochrome <i>c</i> ($\mu\text{mol/g}$)	0.8	0.27	3.0
Cytochrome <i>b</i> ($\mu\text{mol haematin/g}$)	0.64	0.27	2.4
Catalase ($\mu\text{mol haematin/g}$)	0.002	0.034	0.05
Cytochrome <i>a</i> + <i>a</i> ₃ (arbitrary units)	1	0.54	1.9

and also of the amounts of the cytochromes and protohaematin compounds. It should be noted that the actual figures for the succinic dehydrogenase activity have no absolute significance, and can be compared only with one another, not with the succinic oxidase and cytochrome oxidase activities. The cytochrome oxidase activity can, however, be compared with the activity of the complete succinic oxidase system. The activities of the succinic dehydrogenase and of the complete succinic oxidase system are about three times as high in the heart-muscle as in the kidney preparation, and this ratio is similar to the relative intensities of the cytochrome *b* band. This agrees with the usual finding that cytochrome *b* is closely associated with succinic dehydrogenase. The heart muscle preparation has about twice the cytochrome oxidase activity of the kidney preparation and also about twice the content of cytochrome *a* + *a*₃, which agrees with the finding of Keilin & Hartree (1939) that there is a fairly close correlation between the cytochrome oxidase activity and the intensity of the *a* + *a*₃ band.

It has been mentioned above that the figure given for the protohaematin content does not include more than a small fraction of the cytochrome *c*. It does probably include the cytochrome *b*, but the amount of this component is quite insufficient to account for the intensity of the pyridine haemochromogen band. Thus, the total protohaematin content of the heart-muscle preparation is nearly three times that of cytochrome *b*, while the corresponding ratio for the kidney preparation is nearly seven. It is interesting that, although the heart muscle preparation is much richer than the kidney in the cytochromes, both contain about the same concentration of protohaematin compounds. These figures show that both enzyme preparations contain considerable amounts of a haematin compound (or compounds), whose spectrum is not visible either before or after the addition of $\text{Na}_2\text{S}_2\text{O}_4$. One such compound is catalase, but the amount of this enzyme is insufficient to account for more than a very small fraction of the discrepancy between the cytochrome *b* and the total protohaematin content. The kidney preparation contains traces of haemoglobin and some denatured haematin compounds, but insufficient in amount to account for the sixfold discrepancy in that preparation. Certainly, such compounds are not responsible for the discrepancy in the heart muscle preparation, since a carefully made heart muscle preparation shows no signs of haemoglobin or denatured protein compounds.

It is concluded, therefore, that these preparations contain unidentified haematin compounds (or one compound). The fact that the spectra of these compounds are not usually visible in the concentrations in which they are normally found in these preparations suggests that they are not haemochromogens like cytochrome, but resemble compounds of the type of methaemoglobin, catalase or peroxidase, which show only weak absorption bands in either the oxidized or reduced states. That tissues contain a haematin compound in addition to the cytochromes (or even in its absence) was deduced by Keilin (1926, 1929) many years ago on the basis of the great increase in the intensity of the absorption band on the addition of pyridine and reducing agent to many tissues. At first, Keilin believed that the compound was free haematin itself, but he and Hartree (Keilin & Hartree, 1947) have recently shown that haematin is a strong inhibitor of succinic dehydrogenase in low concentrations and have concluded that 'not only the cytochrome compounds, catalase and peroxidase, but all other forms of intracellular haematin exist as compounds with proteins' (see also Keilin & Hartree, 1949). The nature of these haematin compounds must await further investigation. There is evidence (Slater, 1949c) that at least a part of this haematin fraction is probably concerned, like the cytochromes, in the

transfer of electrons from substrate to molecular oxygen.

The point has been made above that the cytochrome system of kidney preparation does not differ in any important respect from that of the heart muscle preparation. Similarly, it seems that there are only minor differences in the succinic oxidase systems. It is shown in another paper (Slater, 1949c), that both tissues require a factor for the reduction of cytochrome *c* by cytochrome *b*. One point of difference between heart muscle and kidney preparations is that the endogenous cytochrome *c* of the kidney preparation is relatively less active than that in the heart muscle preparation. Thus, Fig. 3 shows that the addition of $6 \times 10^{-5}\text{M}$ cytochrome *c* to the heart muscle preparation increased the activity by about 50% (this is a greater increase than found with most heart muscle preparations, the usual increase is only about 20–30%), while the same amount of cytochrome *c* had a much greater effect on the kidney preparation, whose activity was increased about 300%. It is important to note that the concentration of cytochrome *c* necessary for maximum activity ($4 \times 10^{-5}\text{M}$) is very much higher than that actually present in the heart muscle preparation. In fact, the amount of cytochrome *c* added to the manometric flask to give this concentration was 3.3 mg, while the total weight of the heart muscle preparation was usually only 1.2 mg. This illustrates the point already made by Keilin (1930) and Keilin & Hartree (1945, 1949) that added cytochrome *c* is very much less effective catalytically than the cytochrome *c* present in the heart muscle preparation, where it is presumably attached to the particles in such a way that it is more readily accessible to the remainder of the system. This question is also discussed in another paper (Slater, 1949a).

Succinic dehydrogenase and cytochrome b

The suggestion has been made by Bach *et al.* (1946) and by Ball, Anfinsen & Cooper (1947) that cytochrome *b* and succinic dehydrogenase are identical. To avoid confusion, it is necessary to distinguish between two meanings which are given to the term 'succinic dehydrogenase'. Strictly speaking, succinic dehydrogenase means the enzyme which is specifically concerned in the activation of succinate, however, it is often used to refer to that part of the succinic system which is concerned in the catalysis of the reduction of methylene blue by succinate. The following experiment was carried out to test the possibility that cytochrome *b* is part of succinic dehydrogenase, in this latter sense.

A heart-muscle preparation (2 ml) was placed in the main compartment of a modified Thunberg tube, with a side arm (described by Keilin & Hartree, 1947), 0.1 ml 0.003M-sodium succinate was placed in the side arm, and 0.3 ml 0.001M methylene blue

in the hollow stopper. After evacuation, the succinate was added to the heart muscle and the spectrum observed with a low dispersion microspectroscope. The spectrum was the same as that obtained with a higher concentration of succinate except that the *b* band was a little weaker. On adding the methylene blue, the *b* band immediately almost completely disappeared while the *c* and *a* + *a*₃ bands remained visible. The methylene blue was only partially reduced, but its spectrum did not seriously obscure that of the cytochromes. When 2,6-dichlorophenol-indophenol was used instead of methylene blue, the dye was immediately decolorized and the *b* band immediately disappeared. These observations strongly suggest that cytochrome *b* is involved in the reduction of methylene blue and 2,6-dichlorophenol-indophenol by succinate. It is impossible at present to state whether cytochrome *b* is directly reduced by succinate or if this reduction requires an additional enzyme, which is the true succinic dehydrogenase. The solution of this question must await the isolation of either succinic dehydrogenase or cytochrome *b*, which can then be examined to see if it has the properties of the other.

DISCUSSION

Both the heart muscle and kidney preparations are largely composed of small particles in colloidal solution, possessing a very high enzymic activity. Keilin & Hartree (1938, 1939, 1940) have pointed out that such preparations behave in many respects like the living cell. In particular, the respiration is affected by inhibitors in the same way as is the respiration of the living cell. These preparations, especially the more active one from heart muscle, are therefore very suitable for the study of many problems concerned with intracellular respiratory catalysis. The absence of a cell wall eliminates difficulties associated with diffusion through such a barrier, but it has been suggested (Slater, 1949*a*) that problems of diffusion, especially of large molecules, cannot always be dismissed when working with such a preparation. Keilin & Hartree (1949) have shown that the physical properties of these preparations are of paramount importance and must be carefully controlled in studies of the action of inhibitors. This point is further examined in another paper (Slater, 1949*b*).

It might be expected that the drastic mechanical treatment given, especially to the heart muscle, viz. grinding with sand in a mortar for 2 hr., would completely destroy the organization which exists within the living cell. However, this cannot be the case, since the particles have a very high enzymic activity, even of the complex succinic oxidase system (the activity of which is, in fact, much higher in the heart muscle preparation than in the washed minced heart muscle). An active succinic oxidase system must depend on a considerable degree of

organization, with each component situated in the correct spatial relationship to the component with which it reacts. It seems preferable, then, to regard these enzyme preparations as being solutions of some subcellular structure or macromolecules, rather than of unspecific fragments of the mass of the protoplasm.

Keilin & Hartree (1939) have pointed out that the heart muscle preparation contains a considerable amount of copper. This was confirmed in the present investigation. The total copper content of the heart-muscle preparation was found to be 0.0123%, a figure very similar to the 0.0129% of non-dialysable copper found by Keilin & Hartree (1939). This is equivalent to 1.9 micromol of copper/g of fat-free heart muscle preparation, which is the same order of magnitude as the total protohaematin content. It seems not unlikely that this copper is in the form of some catalytically active protein compound.

The Q_{50} , at 37°, of the cytochrome *c* in the heart muscle preparation may be calculated from the figures in Table 1. For purposes of this calculation, it should be noted that the value for the succinic oxidase activity in Table 1 was obtained by measuring the rate of oxidation of succinate in the presence of excess cytochrome *c*. In the absence of added cytochrome *c* the value is about 20% lower, from this figure the Q_{50} of cytochrome *c* at 37° in the heart muscle preparation is 38,000. This is about half the value found by Keilin & Hartree (1940) for yeast, viz. 80,000. The discrepancy is not surprising, since one would hardly expect to have retained, in the heart muscle preparation, the complete organization existing in the living cell. That the factor is as low as 2 indicates that a high degree of organization is retained.

The activity of the cytochrome oxidase is five times that of the succinic oxidase system. This is not unexpected since, in the cell, the oxidation of many substrates, in addition to succinate, passes through the cytochrome oxidase system.

SUMMARY

1. Methods of obtaining, from heart muscle and kidney, enzyme preparations which are suitable for the study of the succinic oxidase system and for the spectroscopic study of the cytochromes are described.

2. The factors involved in the measurement of the succinic dehydrogenase activity and of the complete succinic oxidase system have been investigated.

3. Kidney possesses essentially the same cytochrome system as is found in heart muscle. There is no evidence that cytochrome *b*₁ replaces cytochromes *b* and *c* in the kidney.

4. Quantitative measurements of the amounts of haematin compounds in the enzyme preparations suggest that both contain unknown haematin compounds, whose spectra are not normally visible.

5 Evidence is produced suggesting that cytochrome *b* is involved in the catalysis of the reduction of methylene blue by succinate

6 The enzyme preparations are colloidal solutions of particles, which are probably derived from some subcellular structure in the tissue

7 The fact that the molar concentration of copper in the heart muscle preparation is of the same order of magnitude as that of the haematin

compounds suggests that a copper protein compound might have some catalytic function in the preparation

8 The Q_{O_2} (at 37°) of the cytochrome *c* in the heart muscle preparation is 38,000, i.e. about half the value in yeast

I wish to express my deep appreciation of the most valuable advice given by Prof D Keilin, F.R.S., in the course of this investigation. I also wish to thank the British Council for a scholarship

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The Action of Inhibitors on the System of Enzymes which Catalyse the Aerobic Oxidation of Succinate

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(Received 22 October 1948)

As has been discussed in the previous paper (Slater, 1949a), the enzyme preparations used in the investigation of the succinic oxidase system are colloidal solutions of particles, which are probably derived from some subcellular structure in the tissue. The oxidation of succinate by molecular oxygen requires the co-operation of a number of electron or hydrogen carriers, which appear to be firmly attached to these particles. It is obvious that any one carrier will be unable to react with the carriers before and after it in the reaction chain unless the three are closely situated in or on the particle. Thus, the maintenance of the structure of the particle, which keeps these carriers in the correct spatial relationship to one another, is of paramount importance for the activity of the succinic oxidase system, as has been pointed out by Keilin & Hartree (1940, 1949).

Substances which combine with specific groups in the enzyme molecule, thereby inhibiting the reaction catalysed by the enzyme, are widely used for studying the properties of enzymes. When dealing with a complex system of the type of the succinic oxidase system, the possibility must be considered that the inhibitor does not combine with any specific

groups on the enzyme molecule, but acts non-specifically on the enzyme system as a whole, by affecting the properties of the particles in the solution in such a way as to impair the mutual accessibility of the components of the system. Thus, before inhibitors can profitably be used to obtain information about the components of the succinic oxidase system, the characteristics of the action of inhibitors of this latter type must be determined.

METHODS

The methods of obtaining the enzyme preparations and of measuring enzymic activities have already been described (Slater, 1949a). Except where otherwise stated, the succinic oxidase activity was measured in the presence of added cytochrome *c* (4×10^{-5} M). All activities are expressed as Q_{O_2} (μ l O_2 /mg fat-free dry wt/hr). Most of the measurements of succinic dehydrogenase activity were made by the manometric method used in the previous paper, but in some cases the Thunberg procedure was used.

Denatured globin (kindly supplied by Dr E F Hartree) had been prepared by the method of Keilin & Hartree (1947). $Ca_3(PO_4)_2$ gel was prepared according to the method of Keilin & Hartree (1938).

RESULTS

Effect of ageing

The effect of keeping a preparation of heart muscle at 4° for several weeks on the activities of the succinic dehydrogenase and of the complete succinic oxidase system is shown in Fig 1. It must be

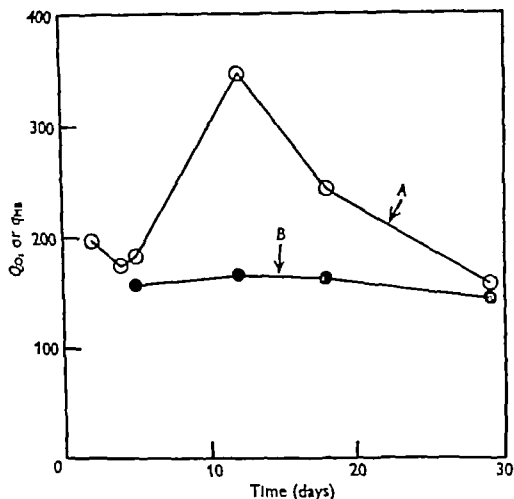


Fig 1 Effect of storage of heart-muscle preparation at 4° on the activities of the succinic oxidase and of succinic dehydrogenase, A, succinic oxidase activity, expressed as Q_{O_2} , B, succinic dehydrogenase activity, determined by the Thunberg method and expressed as q_{MB} , which is in arbitrary units and cannot be compared with the Q_{O_2} .

emphasized that this preparation was not a normal one, probably too much acid was added during the preparation, and consequently its initial succinic oxidase activity was only about one quarter that of a normal preparation. However, the figures are presented because they show very clearly how fluctuations in the activity of the succinic oxidase system are not reflected by the succinic dehydrogenase activities. Usually, the activity of the succinic oxidase system declines after about a week's storage, at which point there is heavy bacterial contamination, while the succinic dehydrogenase activity declines more slowly. This might be due either to the destruction of a component of the succinic oxidase system not required for the succinic dehydrogenase or to changes in the physical properties of the particles. In the present case, however, the increase in the succinic oxidase activity between the first and second week, without change in the succinic dehydrogenase activity of the preparation, must be due to a physical factor. Such an increase in the activity of the succinic oxidase system after 5 days was quite unusual, but an increase during the first day, without a concomitant increase in the succinic dehydrogenase

activity, was quite common. Elliott & Grieg (1938) also found a spontaneous increase in the succinic oxidase activity of a tissue homogenate after standing in the cold for several hours, and attributed this to further dispersion of the components of the succinic oxidase system.

A preparation which was suspended in about one tenth of its volume of M phosphate buffer and glycerol (final concentration, 50 %) did not show any obvious signs of bacterial contamination after a year at 4°. However, its succinic oxidase activity declined, after a few weeks' storage, at about the same rate as that of a normal preparation which was heavily contaminated after about a week. Its succinic dehydrogenase activity, on the other hand, remained constant for at least 3 months.

Effect of phosphate concentration

The effect of phosphate concentration on the activity of the succinic oxidase system and on the succinic dehydrogenase activity of a heart muscle preparation is shown in Fig 2. It can be seen that the

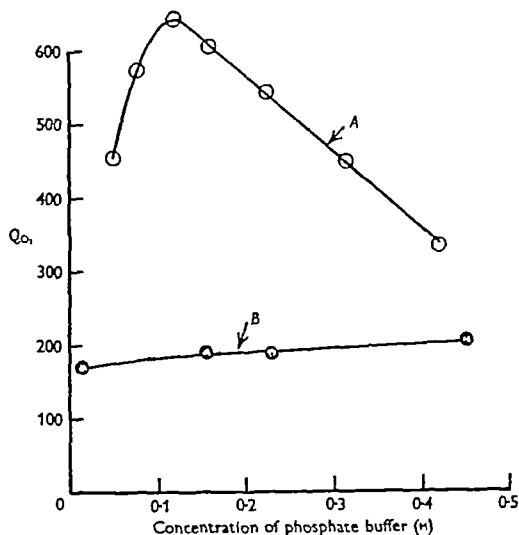


Fig 2 Effect of phosphate concentration on the activity of the succinic oxidase system and of succinic dehydrogenase, A, succinic oxidase system, B, succinic dehydrogenase.

concentration of phosphate buffer is quite critical for the activity of the succinic oxidase system, but has little effect on the succinic dehydrogenase activity. The optimal concentration of phosphate buffer for succinic oxidase activity was 0.11M (cf Keilm & Hartree, 1949, who found that 0.15M was the optimal phosphate concentration). Readings obtained with concentrations of phosphate lower than about 0.03M were somewhat erratic.

If the action of phosphate is physical and affects

the mutual accessibility of the components of the succinic oxidase system, one would expect, since cytochrome *c* is one of these components, that the effect of phosphate would be greater if the succinic oxidase activity were measured in the absence of added cytochrome *c* than in its presence. For example, Keilin & Hartree (1940) showed that certain treatments (e.g. alternate freezing and thawing in liquid air) of the heart muscle preparation, while not affecting the amount of cytochrome *c* in the heart-muscle preparation, did affect its accessibility to the remainder of the succinic oxidase system, so that the activity measured in the absence of added cytochrome *c* was greatly reduced, although the activity in the presence of added cytochrome *c* was not affected. More recently, Keilin & Hartree (1949) have shown that denatured globin can reverse the effect of various physical treatments of the heart-muscle preparation. Table 1 shows the influence of added cytochrome *c* and globin, both separately and together, on the effect of phosphate on the succinic oxidase activity.

Table 1 *Effect of denatured globin and cytochrome c on the effect of phosphate on the succinic oxidase activity of heart muscle preparation*

Globin added Cyt <i>c</i> added Phosphate conc (M)	Succinic oxidase activity (Q_{O_2})			
	Nil	Nil	0.13%	0.13%
	Nil	$4 \times 10^{-5} M$	Nil	$4 \times 10^{-5} M$
A 0.138	467	604	520	695
B 0.028	124	349	595	716
B/A	0.27	0.58	1.14	1.03
C 0.372	323	359	345	414
C/A	0.69	0.65	0.66	0.60

It is apparent from Table 1, first, that the inhibiting effect of low phosphate concentrations is, in the absence of globin, considerably less in the presence than in the absence of cytochrome *c*, and, secondly, in agreement with Keilin & Hartree (1949), that globin completely abolishes the effect of low phosphate concentrations. There is, therefore, reason to believe that the inhibiting action of low phosphate concentrations is due to an effect on the particles of the enzyme preparation, which causes an impaired mutual accessibility of the components of the succinic oxidase system. The inhibiting effect of high phosphate concentrations is not, however, affected by either globin or cytochrome *c*. It is shown in another paper (Slater, 1949b) that high phosphate concentrations have a true and very strong inhibiting action on the cytochrome oxidase. It is possible that the inhibition of the succinic oxidase system by high phosphate concentrations is due to this effect on cytochrome oxidase, which is a part of the succinic oxidase system. Denatured globin has no effect on this inhibition of cytochrome oxidase, nor, of course, has cytochrome *c*, which is the actual substrate of

the enzyme. The variations in cytochrome oxidase activity also found with low phosphate concentrations probably play no part in the effect of such concentrations on the succinic oxidase system, since, in dilute phosphate, the cytochrome oxidase activity is so much higher than that of the succinic oxidase system.

The succinic oxidase activity of the kidney preparation used in the present investigation was affected by the phosphate concentration in much the same way as that of the heart muscle. In this respect, as in others (Slater 1949a), this kidney preparation differed from that of Keilin & Hartree (1949), who found that the activity was optimal at low phosphate concentrations. This difference is probably due to the larger amounts of denatured proteins in the Keilin & Hartree (1949) preparation, since the heart muscle preparation behaves like their kidney preparation if denatured proteins are added.

Effect of surface active compounds

Straub (1942), by dispersing a heart muscle preparation with bile salts, followed by ammonium sulphate fractionation, obtained a preparation which possessed an active succinic dehydrogenase and cytochrome oxidase, but which was unable to oxidize succinate aerobically. He found that the succinic oxidase activity was restored when he added a preparation made by heating heart muscle preparation at pH 9.0 to 55° for 15 min, which treatment completely destroyed the succinic dehydrogenase. He believed that this second preparation contained a factor, the 'SC factor', which linked the succinic dehydrogenase to the cytochrome system, and which was split off the enzyme by the action of bile salts. The experiments of Straub (1942) were repeated, four preparations being made as follows:

(1) *Phosphate extract* Washed minced heart muscle was ground for 2 hr in a mechanical mortar with sand and an equal volume of 0.1 M phosphate buffer, pH 7.3, and centrifuged for 20 min at 2000 rev./min. The supernatant was used.

(2) *Cholate preparation* The method followed was exactly that described by Straub (1942). To 200 ml of the phosphate extract, 50 ml 10% sodium cholate, followed by 43.7 g $(NH_4)_2SO_4$, were added. After standing overnight at 4°, the precipitate was centrifuged off and discarded. The supernatant was treated with 125 g $(NH_4)_2SO_4/l$ and the solution centrifuged. The sticky precipitate, which collected at the top of the centrifuge tube, was dissolved in 30 ml 0.1 M phosphate buffer to give a turbid solution containing 42.6 mg fat-free dry material/ml.

(3) *SC factor preparation* This was also prepared exactly as described by Straub (1942). The phosphate extract (50 ml) was brought to pH 9 by the addition of N NaOH and heated at 55° for 15 min. The mixture was cooled and neutralized with N acetic acid. Weight of the fat-free dried material = 14.4 mg/ml.

(4) *Keilin & Hartree (1947) preparation* This was prepared from the phosphate extract by the method of Keilin & Hartree (1947). Weight of fat-free dried material = 32.3 mg/ml.

The succinic dehydrogenase and succinic oxidase activities of the preparations are compared in Table 2. The cholate preparation had no succinic oxidase activity and its succinic dehydrogenase activity was only about one fifth that of the phosphate extract and one sixth that of the Keilin & Hartree (1947) preparation. The properties of both

Table 2 *Activity of Straub and Keilin & Hartree preparations*

(Activities based on the weight of the fat-free dried substance)

Preparation	Q_{O_2}	
	Succinic oxidase	Succinic dehydrogenase
Phosphate extract	330	150
Cholate preparation	1	33
SC factor	0	1
Keilin Hartree	510	200

the cholate and the SC factor preparations changed on standing for a few days. When both were freshly prepared, the SC factor had a considerable activating effect on the succinic dehydrogenase of the cholate preparation, and also restored the succinic oxidase activity to a small extent (Table 3). After standing

Table 3 *Effect of addition of SC factor to cholate preparation on activity of latter, using fresh preparations*

(Activities expressed in terms of weight of fat-free dried cholate preparation)

SC factor added (mg /mg cholate preparation)	Q_{O_2}	
	Succinic oxidase	Succinic dehydrogenase
0	1	33
0.8	2	72
1.7	14	—

Table 4 *Effect of addition of SC factor to cholate preparation on activity of latter, after keeping both preparations at 4° for 2 days*

(Activities expressed in terms of weight of fat-free dried cholate preparation.)

SC factor added (mg /mg cholate preparation)	Q_{O_2}	
	Succinic dehydrogenase	Succinic oxidase
0	41	2
1.7	48	6
3.4	50	20
6.8	43	29

2 days at 4°, the succinic dehydrogenase activity of the cholate preparation had increased somewhat and the SC factor preparation was less effective in increasing this activity (Table 4). It should be noted that this behaviour of the cholate and SC factor preparations on storage is the exact reverse of that

found by Keilin & Hartree (1949), who found that the activity of the SC factor—obtained by a method slightly different from that of Straub (1942)—increased and the dehydrogenase activity of the cholate preparation decreased after standing a few days. Since the activity of these preparations depends on physical factors which are little understood, it is not surprising that such unpredictable variations should occur. The fact that treatment with bile salts affects, not only the complete succinic oxidase system, but also the succinic dehydrogenase, and that the SC factor preparation reactivates both, indicates that the bile salts do not act specifically on one component of the system, but have a general effect on the whole enzyme system, it is not surprising that its effect on the more complex complete succinic oxidase system should be greater than on the succinic dehydrogenase. Straub's (1942) SC factor must act by reversing this general effect, and not by supplying a missing factor, since it is difficult to see how a factor which operates between succinic dehydrogenase and cytochrome oxidase could re-activate the succinic dehydrogenase.

This conclusion agrees with that of Keilin & Hartree (1949), who showed that the SC factor is not a specific reactivator of the succinic oxidase system treated with bile salts, since they found that it could be replaced by calcium phosphate, which removed the bile salts from the particles. It seems, therefore, that the action of bile salts is due not to the removal of a specific factor, but to a physical effect on the particles of the heart muscle preparation. Such an effect is quite obvious to the eye, since the cloudy colloidal solution of heart muscle is considerably clarified by the addition of bile salts. Bile salts are strong denaturing agents and the strong shading between the *b* and *c* bands observed spectroscopically after the addition of sodium dithionite ($Na_2S_2O_4$) to the cholate preparation is probably due to denatured haematin compounds. The intensities of the *c* and *b* bands were lower than in the heart muscle preparation, although the *a* band was normal.

The action of another surface active agent, viz the haemolytic substance which has been isolated by Laser & Friedmann (1945) from a number of tissues, has also been examined. This substance, which was kindly provided by Dr Laser, is a long-chain unsaturated fatty acid. Its effects are shown in Table 5, which summarizes three separate experiments, Exp 1 with one heart muscle preparation and Exps 2 and 3 with a different preparation. Exps 1 and 2 show that low concentrations of the haemolytic substance practically completely inhibit the succinic oxidase system without having any effect on the succinic dehydrogenase. The two heart muscle preparations differed in their susceptibility to the inhibitor. The effect of the addition of cytochrome *c* on the degree of inhibition of the succinic oxidase system was

Table 5 *Effect of haemolytic substance (HS) on the succinic oxidase system*

	Inhibition (%)			
	Succinic oxidase system		Succinic dehydrogenase	Cytochrome oxidase
	Without cyt c	With cyt c ($6 \times 10^{-5} M$)		
Exp 1 (0.012 ml heart-muscle preparation/ml)				
HS ($1 \times 10^{-4} M$)	—	78	0	—
HS ($1 \times 10^{-4} M$) + 2 mg $Ca_3(PO_4)_2$ /ml *	—	52	—	—
HS ($1 \times 10^{-4} M$) + 0.2 ml. 'SC factor'/ml *	—	6	—	—
Exp 2 (0.012 ml heart-muscle preparation/ml)				
HS ($0.5 \times 10^{-4} M$)	10	11	—	—
HS ($1 \times 10^{-4} M$)	28	23	0	—
HS ($1.75 \times 10^{-4} M$)	60	44	—	—
HS ($2.5 \times 10^{-4} M$)	98	91	1	—
HS ($5 \times 10^{-4} M$)	100	100	—	—
Exp 3 (0.006 ml heart-muscle preparation/ml)				
HS ($1.25 \times 10^{-4} M$)	97	97	26	31
HS ($2.5 \times 10^{-4} M$)	—	—	—	72
HS ($4.5 \times 10^{-4} M$)	—	—	42	—

* The calculated percentage inhibition has been corrected for the stimulating effect of calcium phosphate gel and 'SC factor' preparation on untreated heart-muscle preparation and for the slight residual activity of the 'SC factor' preparation

studied in Exp 2, the results, which are plotted in Fig 3, showing that the inhibition is greater in the absence of added cytochrome c than in its presence. The S shape of the curves in Fig 3 is probably due

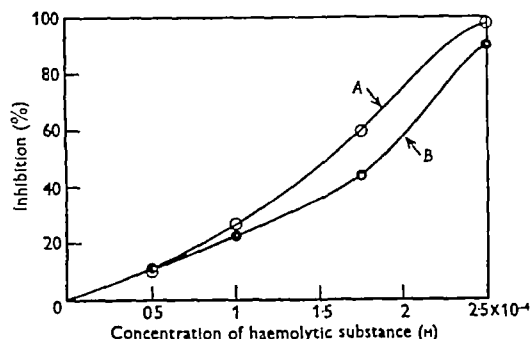


Fig 3 Effect of adding cytochrome c ($6 \times 10^{-5} M$) on the inhibition of the complete succinic oxidase system by Laser's haemolytic substance (HS), A, percentage inhibition of system, measured in the absence of added cytochrome c, B, percentage inhibition of system, measured in the presence of added cytochrome c

to the protective action, at low concentrations of the inhibitor, of other substances (e.g. denatured proteins) in the enzyme preparation. This is probably also the reason for the finding in Exp 3 that when the concentration of the enzyme preparation was halved it became much more susceptible to the inhibitor. In Exp 3, it was found that succinic dehydrogenase and cytochrome oxidase were both inhibited to a certain extent by a concentration of inhibitor sufficient completely to inactivate the complete succinic oxidase system. It is apparent from Exp 3 that the order of increasing susceptibility to

the inhibitor is succinic dehydrogenase, cytochrome oxidase, complete succinic oxidase system.

Both calcium phosphate gel and Straub's (1942) SC factor were able to reactivate the succinic oxidase system after treatment with the haemolytic substance, reactivation by the SC factor preparation was complete (Exp 1). It seems very likely that this haemolytic substance acts on the succinic oxidase system in the same manner as bile salts.

DISCUSSION

In considering the action of inhibitors on the succinic oxidase system, it is advisable to distinguish between those substances which act specifically on one component of the system (e.g. malonate on succinic dehydrogenase, cyanide on cytochrome oxidase) and those which act non-specifically on the enzyme system as a whole, by affecting the mutual accessibility of the components of the system, on the particles of the enzyme preparation (cf. Keilin & Hartree, 1949). Examples of this non-specific type of action are adsorption of the inhibitor on the particle, dispersal by surface active substances, and aggregation. Such treatments must have a more drastic action on the complete succinic oxidase system (involving a number of components) than on those reactions which depend upon only some of the components of the system, for example the reduction of methylene blue by succinate. Thus, it has been invariably found that, with these types of inhibitors, the complete succinic oxidase system is much more susceptible than is the succinic dehydrogenase, in fact, concentrations sufficient to inhibit completely the succinic oxidase system often have no effect on the succinic dehydrogenase, which

require higher concentrations in order to detect any inhibition. The susceptibility of the cytochrome oxidase to the haemolytic substance is intermediate between that of the succinic dehydrogenase and the complete system.

Inhibitors which act in this non specific manner possess certain characteristics which will usually enable them to be distinguished from the more specific inhibitors, viz (1) concentrations which are necessary to inhibit completely the succinic oxidase system will affect the cytochrome oxidase to a certain extent, while higher concentrations will have a strong effect on the cytochrome oxidase, and, perhaps, some effect on the succinic dehydrogenase, (2) the inhibition of the complete succinic oxidase system is lower when the activity of the latter is measured in the presence of cytochrome *c* than when it is measured in its absence, (3) the inhibition is often reversed by the addition of substances such as calcium phosphate gel or denatured proteins, which either remove the inhibitor from the particles by absorption (as is probably the case with surface-active compounds) or, in some unknown way, are able to restore the mutual accessibility of the components of succinic oxidase system on the particles after the latter have been affected by physical agents (for example, effect of globin on the effect of low phosphate concentrations).

The more specific inhibitors will, generally, act on only one component of the system, will be unaffected by the presence or absence of cytochrome *c* (unless, of course, the inhibitor acts specifically on the cytochrome *c*, no such inhibitor is, however, known) and will be unaffected by the addition, after the inhibitor has acted on the enzyme, of substances such as denatured proteins or calcium phosphate gel. Denatured globin can, however, reverse the action of substances which combine with thiol groups (Slater, 1949c).

If all these points are considered, together with the general physical and chemical properties of an

inhibitor, it should be possible, in most cases, to determine whether the inhibitor has a general non-specific effect on the system or is acting specifically on a single component of the system. A particular application of this method of distinguishing between the two types of inhibition will be found in the following paper (Slater, 1949d).

SUMMARY

1 During storage of a particular heart muscle preparation at 4° for several weeks, the activity of the succinic oxidase system at first increased, then decreased, while the succinic dehydrogenase activity remained constant. It is believed that this is due to an alteration of the physical properties of the particles of the enzyme preparation.

2 The concentration of the phosphate buffer has a profound effect on the activity of the succinic oxidase system, but has little action on the succinic dehydrogenase. The inhibitory action of low phosphate concentrations was greater when the activity was measured without the addition of cytochrome *c*, it was completely prevented by denatured globin.

3 Straub's (1942) claim to have removed, by the action of bile salts, a component of the succinic oxidase system, which can be restored by the addition of a heart muscle extract, heated under alkaline conditions, has not been confirmed. Bile salts have a general physical effect on the particles of the enzyme preparation.

4 A haemolytic substance had the same effect as bile salts on the system.

5 Inhibition of the succinic oxidase system by non specific agents possesses certain characteristics, which distinguishes this inhibition from that induced by agents which combine specifically with a component of the system.

I wish to thank Prof D Keilm, F.R.S., for his interest and advice during this investigation, and the British Council for a scholarship.

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A Respiratory Catalyst Required for the Reduction of Cytochrome *c* by Cytochrome *b*

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(Received 22 October 1948)

For the study of the cytochromes, the enzyme system catalysing the aerobic oxidation of succinate (the succinic oxidase system) offers special advantages since, unlike most intermediary metabolites, succinate activated by its dehydrogenase reacts directly with the cytochrome system without the necessity for the intermediary carriers cozymase and diaphorase. The work of Keilin (1929), Keilin & Hartree (1940) and Ball (1938) shows that the transport of hydrogen atoms (or electrons) from succinate to molecular oxygen proceeds through a chain of electron carriers of successively higher oxidation reduction potential. Four such carriers have been identified spectroscopically, acting in the following manner, the arrows indicating the direction of electron transfer (cyt = cytochrome)

Succinate \rightarrow cyt *b* \rightarrow cyt *c* \rightarrow cyt *a* \rightarrow cyt *a*₃ (oxidase) \rightarrow O₂

|
Succinic dehydrogenase

E'_0 at pH 7.3 and 30°

-0.010* -0.04† 0.262‡ 0.29‡ ? 0.78 V

The activity of the complete succinic oxidase system is measured by determining the rate of oxygen uptake in the presence of excess succinate, while the component parts of the system may be studied by the introduction of substances of suitable oxidation reduction potential which do not require activation by enzymes. Thus the rate of reduction of methylene blue ($E'_0 = +0.001$ V, at pH 7.3, 30°, Clark, Cohen & Gibbs, 1925) in the presence of excess succinate is a measure of the succinic dehydrogenase cytochrome *b* portion of the system, which will henceforth be referred to as 'succinic dehydrogenase' (It is possible that succinic dehydrogenase is identical with cytochrome *b*. See Bach, Dixon & Zerfas, 1946, Ball, Anfinsen & Cooper, 1947, Slater, 1949a). Similarly, the activity of the cytochrome *a* cytochrome *a*₃ portion (henceforth referred to as cytochrome oxidase) may be measured by determining the rate of oxidation, in the presence of excess cytochrome *c*, of a number of substances, such as *p*-phenylenediamine, ascorbic acid and quinol, which rapidly reduce cytochrome *c*.

Several groups of workers have suggested that the aerobic oxidation of succinate requires an additional factor acting between succinic dehydrogenase and cytochrome *c*. This suggestion was based upon the evidence that it was possible, by various treatments, to obtain enzyme preparations which were able to catalyse the reduction of methylene blue by succinate and the oxidation of reducing agents in the presence of cytochrome *c*, and yet were unable to oxidize succinate aerobically. Such preparations can be obtained by treatment with bile salts (Hopkins, Morgan & Lutwak Mann, 1939, Straub, 1942), repeated isoelectric precipitation or ultracentrifugation (Stern & Melnick, 1939), or by ammonium sulphate fractionation (Stoppani, 1947). Keilin & Hartree (1940) obtained a similar preparation by treatment of the enzyme preparation at pH 5 for 1 hr, but suggested that the inactivation of the succinic oxidase system might be due, not to the destruction or removal of a factor of the type suggested by the other workers, but to an effect on the particles of the enzyme preparation which caused an impaired accessibility of the components of the succinic oxidase system, without appreciably affecting the accessibility of methylene blue or succinate to the dehydrogenase. Stern & Melnick (1939), Straub (1942) and Stoppani (1947) appeared to have obtained strong evidence in support of their view, by restoring the activity of the complete system by the addition of alleged specific factors such as the supernatant fluid from the ultracentrifugation (Stern & Melnick, 1939), an enzyme preparation heated to 55° at pH 9 (Straub, 1942) or a preparation obtained by fractional precipitation with ammonium sulphate (Stoppani, 1947). Keilin & Hartree (1949) have now shown, however, that these supposed specific reactivating fractions could be replaced by such substances as calcium phosphate gel or denatured proteins, which could not possibly be components of the succinic oxidase system. It follows that the enzyme preparations obtained by Stern & Melnick, Straub and Stoppani must have contained all the components of the succinic oxidase system, and that the inactivity must have been due to the loss of the mutual accessibility of these components. The reactivating substances probably act, in some as yet unexplained manner, by restoring this accessibility.

* Calculated from Borsook & Schott (1931)

† Ball (1938)

‡ Stotz, Sidwell & Hogness (1938)

However, Keilin & Hartree (1949) admit that, although their experiments disprove the claims of these workers, they do not disprove the possible existence of such a factor. One method of investigating this possibility is to search for substances which inhibit the complete succinic oxidase system without affecting the activities of the succinic dehydrogenase, cytochrome *c* or cytochrome oxidase, and which do not act non-specifically on the enzyme system as a whole by affecting the particles of the enzyme preparation. In another paper (Slater, 1949*b*), ways of distinguishing between inhibitors which have this latter kind of action and those which act specifically on a component of the system are discussed. The present paper is concerned with the finding that certain reducing agents, in the presence of air, bring about complete inactivation of the succinic oxidase system, without affecting either the succinic dehydrogenase or the cytochrome oxidase. A detailed study of the mechanism of this inactivation showed that it was due to the destruction of a specific component of the succinic oxidase system. This component, which is probably a haematin compound, acts in the system between cytochrome *b* and cytochrome *c*.

A preliminary account of the main findings of this investigation has been given elsewhere (Slater, 1948)

EXPERIMENTAL

Enzyme preparations from horse heart and kidney, obtained by the methods previously described (Slater, 1949*a*), were used as the succinic oxidase system.

Cytochrome c, prepared by the method of Keilin & Hartree (1945*a*), contained 0.34% Fe, *catalase* was prepared by the method of Keilin & Hartree (1945*b*) and *D-amino-acid oxidase* by the method of Negelem & Brömel (1939), *notatin*

previously described (Slater, 1949*a, c*). All activities are expressed as Q_{O_2} (μ l O_2 /mg fat-free dry wt/hr).

Copper was determined by the method of McFarlane (1932).

General procedure

The effect of the reducing agent on the enzyme system was most conveniently studied by the following procedure, which will henceforth be referred to as the 'general procedure'. Undiluted enzyme preparation (1 ml.) was pipetted into a Barcroft flask, followed by any other additions to be made and finally by the reducing agent, the total volume (made up with glass distilled water) being 2 ml. After attaching to a Barcroft manometer the flask was shaken in air at 37° for the required time, then removed from the manometer, and 3 ml. 0.18M phosphate buffer added. Samples (0.2 ml.) were pipetted into flasks for the measurement of the enzyme activities, and immediately diluted with the appropriate amount of phosphate buffer. This rapid dilution (about 40 fold) almost completely prevented any further reaction between the reducing agent and the enzyme. Controls treated in exactly the same manner, but using water instead of the reducing agent, were always included and used as the basis for calculating percentage inhibitions.

This procedure, namely reaction at high enzyme and inhibitor concentrations followed by dilution before measuring the enzyme activities, is only valid if the inhibition is not reversed by dilution, as was the case with the inhibitions discussed in this paper.

RESULTS

Effect of reducing agents on the activities of the succinic oxidase system and of succinic dehydrogenase

The effects of equivalent concentrations of ascorbic acid and some thiol compounds on the activities of the succinic oxidase system and of succinic dehydrogenase in the heart muscle preparation are shown in

Table 1 *Effect of reducing agents on the activities of the succinic oxidase system and of succinic dehydrogenase in heart muscle preparation*

('General procedure' (see above), reaction time, 30 min.)

Reducing agent	Concentration (M)	Oxygen uptake in 30 min (μ l.)	Inhibition (%)	
			Succinic oxidase system	Succinic dehydrogenase
Ascorbic acid	0.01	47	77	28
Glutathione	0.02	96	78	9
Cysteine	0.02	247	100	79
Diethyldithiocarbamate	0.02	65	100	100
2,3-Dimercaptopropanol (BAL)	0.01	133	98	5

was obtained (by courtesy of Dr W. F. Short) from *Penicillium notatum*.

Glutathione was prepared by the methods of Hopkins (1929) and of Pirie (1930). It was dissolved and neutralized immediately before use.

2,3-Dimercaptopropanol (BAL) was kindly supplied by the Department of Biochemistry, Oxford. Aqueous solutions were prepared with glass distilled water immediately before use.

Enzyme activities were measured according to the methods

Table 1. Diethyldithiocarbamate completely inhibited the succinic dehydrogenase activity (cf Keilin & Hartree, 1940). The other three thiol compounds strongly inhibited the activity of the complete system, but, with the exception of cysteine, had little effect on the succinic dehydrogenase. The degrees of inhibition caused by these three compounds were in the same order as their rates of oxidation in the presence of the heart muscle pre-

paration Ascorbic acid inhibited the complete system to about the same extent as glutathione, but had a greater effect on the succinic dehydrogenase. Under the conditions of this experiment, the compound which had the greatest effect on the complete succinic oxidase system with very little effect on succinic dehydrogenase was 2,3-dimercaptopropanol (BAL). Since it was also found that this compound had very little effect on the cytochrome oxidase activity, it fulfilled the requirements for the present study, and the mechanism of its action was further investigated. These observations reconcile the apparently divergent findings of Webb & van Heyningen (1947) and of Barron, Miller & Meyer (1947) on the effect of BAL on the succinic system, since the latter authors, who found an inhibition, measured the activity of the complete succinic oxidase system, while the former authors, who found no effect, measured the succinic dehydrogenase.

Effect of BAL on the activities of the succinic oxidase system and of succinic dehydrogenase

A study of the effect of time of contact of BAL with the heart muscle preparation before dilution, summarized in Fig. 1, showed that the succinic

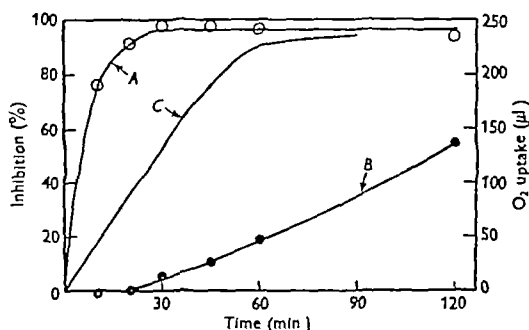


Fig. 1. Inactivation of the succinic oxidase system and succinic dehydrogenase in heart-muscle preparation by shaking in air with 0.0094M BAL at 37°C for various periods of time, 'general procedure' (see p. 15). A, inhibition of succinic oxidase system; B, inhibition of succinic dehydrogenase; C, O₂ uptake by BAL; 0.2 ml. 0.004M BAL added to flasks used for measurement of control activities, i.e. these flasks contained the same amount of BAL (0.00025M) as test flasks. This amount of BAL inhibited the succinic oxidase activity by 11% and the succinic dehydrogenase by 3%.

oxidase system was inhibited after a few minutes' contact with BAL, and the inhibition was 90% after 20 min. Inhibition of the succinic dehydrogenase, on the other hand, did not commence until about 30 min. after the addition of the BAL to the heart muscle preparation, i.e. when 57% of the BAL was oxidized. Thereafter, the inhibition of succinic dehydrogenase increased rapidly with time. It was

found that shaking the heart muscle preparation with air for 2 hr. at 37°C under the conditions of the experiment, but in the absence of BAL, caused no destruction of the succinic dehydrogenase, and only a 13% loss of the succinic oxidase activity.

It is apparent from these experiments that BAL has two quite distinct effects: (1) a rapid and strong inhibition of the succinic oxidase system, without any effect on the succinic dehydrogenase portion, (2) an inhibition of the succinic dehydrogenase, which does not commence until more than half the BAL is oxidized and increases in velocity as the oxidation of BAL approaches completion.

The possibility that the subsequent addition of KCN in the measurement of the succinic dehydrogenase activity might have reversed an inhibition of succinic dehydrogenase, caused by the BAL, was excluded by an experiment in which the inhibition was measured in two ways, viz. by the manometric method in the presence of KCN, and by the Thunberg method in its absence. The calculated inhibitions were the same by both methods.

The experiment described in Fig. 1 suggested that the effect on succinic dehydrogenase, which did not occur until more than half the BAL was oxidized,

Table 2. *Effect of BAL and oxidized BAL on the succinic oxidase and succinic dehydrogenase activity of heart muscle preparation*

(A) Alkali treated preparation (1 ml.) + 1 ml. 0.028M BAL were added to a Barcroft flask which was attached to a manometer and shaken at 37°C until the uptake of O₂ had ceased (60 min.). Heart-muscle preparation (1 ml.) was then added to the flask, which was shaken at 37°C for a further 30 min., 2 ml. 0.18M phosphate buffer were then added and the succinic dehydrogenase and succinic oxidase activities determined on 0.2 ml. samples in the usual way. B This was the same as A, except that 1 ml. water replaced the BAL. C Alkali treated preparation (1 ml.) + 1 ml. 0.028M BAL + 1 ml. heart-muscle preparation were added to a Barcroft flask which was shaken at 37°C for 30 min., 2 ml. 0.18M phosphate buffer were then added and the succinic dehydrogenase and succinic oxidase activities determined on 0.2 ml. samples in the usual way. D This was the same as C, except that 1 ml. water replaced the BAL.)

	Succinic oxidase activity		Succinic dehydrogenase activity	
	μl./10 min	Inhibition (%)	μl./10 min	Inhibition (%)
A	68.6	34	32.1	23
B	104.7	—	41.8	—
C	2.6	98	32.9	20
D	104.2	—	41.0	—

was due to an oxidation product of BAL. Consequently, the effect of oxidized BAL was compared with that of BAL itself. BAL was oxidized by shaking in air with a heart muscle preparation, treated by the method of Straub (1942) in order to

destroy its succinic dehydrogenase activity, until the absorption of O_2 ceased. The treatment of the heart muscle preparation consisted of bringing it to pH 9.0, warming at 55° for 15 min and then cooling and neutralizing. The experiment, described in Table 2, clearly shows that, whereas the inhibition of succinic dehydrogenase activity was about the same when previously untreated heart muscle preparation was in contact with either completely oxidized BAL (A) or with BAL undergoing oxidation (C), the inactivation of the succinic oxidase system was much greater when it was present during the oxidation of the BAL. The oxidized BAL produced a slightly greater inhibition of the succinic oxidase system than of the succinic dehydrogenase, other oxidizing agents behave similarly (Slater, 1949*d*). The effect of oxidized BAL on succinic dehydrogenase is probably due to oxidation of SH groups essential for the activity of the dehydrogenase (cf the inhibition of succinic dehydrogenase by oxidized glutathione, Hopkins & Morgan, 1938).

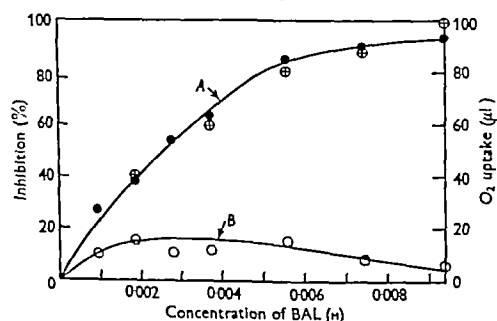


Fig 2 Inactivation of the succinic oxidase system and of succinic dehydrogenase in heart-muscle preparation by shaking in air with various concentrations of BAL, temp 30° , reaction time, 20 min., 'general procedure' A, ● inhibition of succinic oxidase system, B, ○ inhibition of succinic dehydrogenase ⊕ O_2 absorbed by BAL during contact with the heart-muscle preparation (right-hand ordinate scale)

The effect of the concentration of BAL on the inactivation of the succinic oxidase system and of succinic dehydrogenase is shown in Fig 2. The inactivation of the succinic oxidase system increased with increasing concentration of BAL, following the same curve as the amount of O_2 consumed by the BAL during the period of the experiment (20 min). The inhibition of succinic dehydrogenase did not however, increase in the same way, the maximum inhibition was obtained at intermediate concentrations, this effect being more marked when, in other experiments, the time of contact with the BAL was increased. The above findings are understandable in view of the fact that the degree of the inhibition of succinic dehydrogenase is governed by two factors, viz (1) the concentration of the oxidation product of BAL, (2) the proportion of oxidized BAL to total

BAL. With increasing initial concentration of BAL, (1) increases while (2) decreases (see Table 3, the concentration of oxidized BAL after 20 min can be taken to be approximately proportional to the amount of O_2 consumed during the experiment).

Table 3 Effect of concentration of BAL on rate of oxygen uptake of BAL in presence of heart muscle and on the percentage of total BAL in the oxidized state after 20 min

(Heart-muscle preparation (1 ml.), total volume 2 ml., temp 39° , see also Fig 2)

BAL concentration (M)	Initial rate of oxidation of BAL ($\mu l O_2/20$ min)	O_2 absorbed in first 20 min (μl)	Percentage of total BAL in oxidized form after 20 min
0.00186	49	40	92
0.00372	62	60	66
0.00558	85	81	61
0.00774	95	88	51
0.0093	102	99	47

Table 4 Comparison of effect of BAL on succinic oxidase and succinic dehydrogenase systems in heart muscle and kidney preparations

('General procedure', see p 15, reaction time, 20 min., values for heart muscle are plotted in Fig 2)

BAL concentration (M)	Inhibition (%)			
	Succinic oxidase		Succinic dehydrogenase	
	Heart muscle	Kidney	Heart muscle	Kidney
0.00141	29	63	14	—
0.00382	54	85	16	23
0.0094	93	100	6	12

Table 5 Effect of BAL under aerobic and anaerobic conditions on the succinic oxidase activity of heart muscle

(Anaerobic treatment 1 ml of undiluted heart-muscle preparation + 0.8 ml. water were placed in a Thunberg tube and 0.2 ml. 0.095M BAL in the hollow stopper. The air in the Thunberg tube was replaced by O_2 free N_2 , and, after the contents of the tube had been mixed with those of the hollow stopper, the tube was heated for 30 min at 38° . The tube was then cooled in ice, opened to air and 3 ml. cold 0.18M phosphate buffer quickly added. After mixing, 0.2 ml was pipetted into a Barcroft flask and immediately diluted with 2.7 ml 0.18M phosphate buffer. Aerobic treatment same quantities shaken with air at 38° for 30 min., then treated as above. Control treated as 'aerobic treatment', but water replaced BAL. 0.2 ml 0.004M BAL added to Barcroft flask used for measurement of enzymic activity, in order to give same final concentration of BAL as in other flasks.)

	O_2 uptake ($\mu l/10$ min)	Inhibition (%)
Anaerobic treatment	64.4	12
Aerobic treatment	1.2	98
Control	73.1	—

Thus there is a balance between these two opposing factors, with the result that the inhibition is optimal at intermediate concentrations of BAL

The kidney preparation behaved essentially like heart muscle except that it showed a greater susceptibility to low concentrations of BAL (Table 4)

It was found that while BAL produced practically complete inhibition under aerobic conditions, it produced very little inhibition if its oxidation was prevented by working under anaerobic conditions (Table 5) The slight inhibition under anaerobic conditions probably occurred during the final dilution, in air

These experiments show that the effect of BAL on the succinic system is not due to BAL itself or to its oxidation product, but to BAL undergoing aerobic oxidation in the presence of the enzyme system Accordingly, the oxidation of BAL in the presence of the heart muscle preparation was further studied

Oxidation of BAL

The following observations (Table 6) showed that H_2O_2 is produced during the aerobic oxidation of BAL in buffer solutions (1) the total O_2 uptake of BAL in buffer solution was slightly greater than the theoretical uptake, calculated from the equation $2\text{CH}_2\text{OH CHSH CH}_2\text{SH} + \text{O}_2 \rightarrow \text{disulphide compounds} + 2\text{H}_2\text{O}$, (2) this extra O_2 was evolved if catalase was added after the solution had ceased to absorb O_2 , (3) if catalase was present during the oxidation there was no subsequent evolution of O_2 on the addition of catalase

Table 6 Oxygen uptake of BAL under various conditions

(Enzyme preparation or phosphate buffer (0.05M, 1 ml) and BAL ($18.5 \mu\text{M} \equiv 207 \mu\text{l O}_2$) in total volume of 2 ml., temp 37° , catalase added from dangling tube after O_2 uptake ceased)

	Initial rate of O_2 uptake ($\mu\text{l/hr}$)	Final O_2 uptake (μl)	O_2 evolved after addition of catalase (μl)	Net O_2 uptake (μl)	Net uptake (% theoretical)
Phosphate buffer	121	220	8	212	102
Phosphate buffer + catalase*	—	216	0	216	104
Heart-muscle preparation†	298	236	0	236	114
Kidney preparation	230	224	—	—	—

* $1.6 \times 10^{-6}\text{M}$ (mol wt = 240,000)

† These figures have been corrected for the slight endogenous respiration (probably due to bacterial action) measured after the BAL was completely oxidized Treatment with the BAL considerably reduced the endogenous respiration

In the presence of heart muscle preparation, the O_2 uptake considerably exceeded the theoretical value, but there was no evolution of O_2 on the subsequent addition of catalase The formation of H_2O_2 during the oxidation of BAL in the presence of heart muscle preparation was demonstrated by the addition of ethanol and catalase to the system (Fig 3) Whereas catalase or ethanol alone had little effect on the oxidation of BAL, the rate of O_2 uptake and the final uptake were considerably increased when both ethanol and catalase were added Since the heart muscle preparation does not oxidize

ethanol, and since catalase does not do so unless H_2O_2 is formed by some other reaction (Keilin & Hartree, 1945c), it follows that H_2O_2 must have been

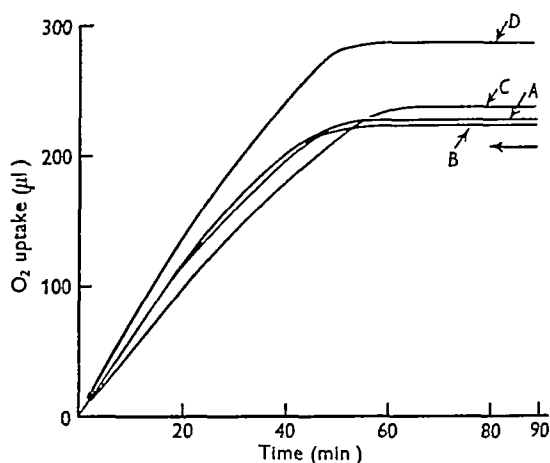


Fig 3 Effect of catalase and ethanol on the O_2 uptake of BAL + heart-muscle preparation All flasks contained 1 ml heart-muscle preparation in a total volume of 2 ml BAL, 0.0094M, temp 37° , pH 7.1 A, no addition, B, $2.6 \times 10^{-6}\text{M}$ catalase, C, 40 mg ethanol, D, $2.6 \times 10^{-6}\text{M}$ catalase + 40 mg ethanol The horizontal arrow indicates the theoretical O_2 uptake for the reaction $2\text{BAL} + \text{O}_2 \rightarrow \text{BALOx} + 2\text{H}_2\text{O}$ The figures have been corrected for the endogenous respiration (see footnote to Table 6)

produced during the oxidation of BAL in the presence of the heart muscle preparation The necessity for

the addition of catalase as well as ethanol, even though the heart muscle preparation contains sufficient catalase to decompose H_2O_2 immediately, is understandable in view of the finding of Keilin & Hartree (1945c) that larger concentrations of catalase are required for the peroxidatic than for the catalytic reaction of catalase The amount of catalase added was 300 times that present in the heart muscle preparation

The fact that the O_2 uptake of BAL in the presence of heart muscle preparation was greater than the theoretical value without any accumulation of H_2O_2

at the end of the oxidation must mean that some substance (or substances) in the heart muscle preparation was oxidized during the oxidation of the BAL.

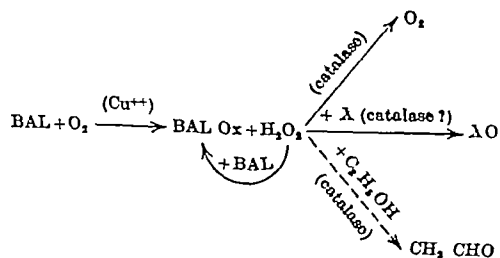
Table 6 shows that the rate of oxidation of BAL was increased 2.5 times by the presence of the heart muscle preparation, and doubled by the presence of the kidney preparation. Since Barron, Miller & Kalnitsky (1947) have reported that copper catalyses the oxidation of BAL, the possibility that this increased rate of oxidation might be largely due to copper in the heart muscle preparation was investigated. It was found by direct analysis that the heart muscle preparation contained sufficient copper (4.2×10^{-5} M) to account for its catalytic effect on the oxidation of BAL, provided that this copper was as effective as Cu^{++} ions added to the preparation.

However, it is probable that the copper in the heart muscle preparations does not arise from accidental contamination with Cu^{++} ions, but is in the form of firmly bound copper protein compounds, the availability of this copper for the catalytic oxidation of BAL is not known. Further experiments, described below, were designed to give more information of this point.

Mechanism of the effect of BAL on the succinic oxidase system

It has been shown above that (1) BAL strongly inhibits the succinic oxidase system only when it is oxidized by air in the presence of the enzyme preparation, and (2) some substance (or substances) in the heart muscle preparation is oxidized during the process of oxidation of BAL in the presence of heart muscle preparation. It seems highly probable that BAL inhibits the succinic oxidase system by causing the oxidation of some substance (X) necessary for the activity of the succinic oxidase system. Two possible schemes (A and B) describing this effect of BAL may be considered.

According to the first scheme (A), BAL is oxidized (by copper catalysis) with the formation of oxidized

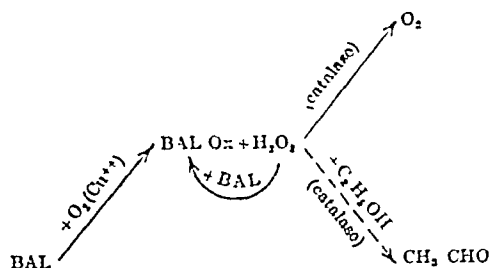


Scheme A

BAL (BAL Ox) and H_2O_2 . The latter may take part in three competing reactions, viz (1) catalytic destruction by the catalase present in the heart muscle

preparation, (2) oxidation of more BAL, (3) oxidation of the compound (X) as well as of some other substances in the heart muscle preparation. The latter reaction may be catalysed by catalase. In the presence of ethanol and additional catalase, a fourth reaction (shown by the dotted line in the diagram) is possible.

In the second scheme (B), BAL is oxidized by two competing reactions, one involving copper catalysis as in Scheme 1 and the other involving a directly coupled oxidation of BAL with X, in which reaction free H_2O_2 , able to react with catalase, is not formed. In this case, as well as in Scheme A, the H_2O_2 formed by the copper catalysed oxidation might also oxidize other substances in the heart muscle preparation.



Scheme B

Either of these schemes could explain the destruction of X, the fact that the O_2 uptake of BAL in the presence of heart muscle preparation exceeds the theoretical, and the extra O_2 uptake in the presence of ethanol and catalase. For the sake of simplicity, a further pathway of oxidation of BAL, viz through cytochrome c and cytochrome oxidase, has been omitted from both schemes. This reaction could not involve X nor could it contribute to the extra uptake of O_2 , since H_2O_2 is not formed during oxidation through cytochrome oxidase. It was calculated from the rate of reduction of the cytochrome c in the heart muscle preparation by BAL under anaerobic conditions (cf a similar calculation for ascorbic acid, Slater 1949c) that the oxidation of BAL through cytochrome c and cytochrome oxidase could account for no more than one quarter of the O_2 uptake catalysed by heart muscle. BAL reduces cytochrome b, as well as cytochrome c, but the rate of this reduction is so low (time of half reduction about 30 min) that the rate of oxidation through cytochrome b would be insignificant.

When sufficient extra cytochrome c is added, the oxidation through the cytochrome oxidase system becomes the main pathway (see p 22).

The experiments, which will now be described, showed that Scheme *A* did not correctly express the reactions involved and that Scheme *B* was probably correct

(1) *Effect of hydrogen peroxide on the activities of succinic dehydrogenase and the succinic oxidase system in heart muscle* According to Scheme *B*, the destruction of *X* is caused by a direct reaction with BAL (in the presence of O_2). According to Scheme *A*, however, the destruction of *X* is not caused directly by BAL, but by H_2O_2 produced by the oxidation of BAL. If Scheme *A* is correct, it should be possible to obtain inhibitions of the same type as BAL by means of H_2O_2 produced in a number of different ways. Accordingly, the effects of two primary reactions which produce H_2O_2 , viz. D amino acid oxidase, with DL alanine, DL methionine and D isoleucine as substrates, and notatin (glucose oxidase) with its substrate glucose, were studied. The results, summarized in Table 7, show that the D amino acid oxidase system produced very little inhibition of the succinic oxidase system. The notatin glucose system, on the other hand, caused some inhibition, but, although the rate of O_2 uptake (and therefore the rate of formation of H_2O_2) was in some experiments less

and in others much greater than in the experiments with BAL, the greatest inhibition was only 40%. The degree of inhibition seemed to be independent of the amount of notatin used. These inhibitions of the succinic oxidase system were accompanied by smaller but considerable inhibitions of the succinic dehydrogenase. The reason for the difference between the D amino acid oxidase and the notatin glucose systems is considered below.

(2) *Effect of catalase and ethanol on the inhibition of the succinic oxidase system produced by notatin glucose and by BAL* If the effect of BAL on the succinic oxidase system is due to the action on *X* of H_2O_2 produced by the oxidation of BAL, as in Scheme *A*, the addition of catalase and ethanol would be expected to protect *X* from oxidation and the succinic oxidase system from inhibition, the degree of protection depending upon the relative affinity of *X* and the catalase ethanol system for H_2O_2 . Table 8 shows the effect of catalase and ethanol on the inhibition of succinic oxidase produced by notatin glucose and by BAL.

In the case of the notatin glucose system, the possible reactions are similar to Scheme *A* and, as would be expected, the addition of ethanol, by com

Table 7 *Effect of hydrogen peroxide produced by aerobic dehydrogenases and their substrates on the activities of the succinic oxidase system and of succinic dehydrogenase in heart-muscle preparation*

('General procedure', see p. 15), reaction time, 30 min., D amino acid oxidase = A. oxid.)

System producing H_2O_2	Initial rate of O_2 uptake (μ l./hr.)	Inhibition (%)	
		Succinic oxidase	Succinic dehydrogenase
A. oxid. (10 mg) + DL alanine (3 mg)*	210	11	—
A. oxid. (50 mg)* + DL-alanine (3 mg)	1000	0	0
A. oxid. (10 mg) + DL-methionine (5 mg)	480	6	6
A. oxid. (10 mg) + D isoleucine (2.4 mg)	38	14	0
Notatin (5 μ g) + glucose (10 mg)*	90	38	12
Notatin (10 μ g) + glucose (10 mg)	190	22	12
Notatin (25 μ g)* + glucose (10 mg)	415	38	24
Notatin (40 μ g) + glucose (10 mg)	570	28	17
Notatin (50 μ g) + glucose (10 mg)	660	40	12
BAL (0.01 M)	315	98	5

* No inhibition by these substances alone

Table 8 *Effect of catalase and ethanol on the inhibition of the succinic oxidase system produced by notatin glucose and by BAL*

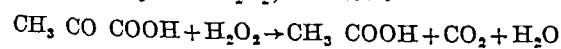
('General procedure', see p. 15)

Addition	Inhibition by		
	0.0031 M BAL, acting for 20 min (%)	0.0094 M BAL, acting for 30 min (%)	Notatin (25 μ g) + glucose (10 mg), acting for 30 min (%)
None	53	99	37
0.35 M Ethanol*	66	—	26
2.6×10^{-6} M Catalase*	53	97	61
2.6×10^{-6} M Catalase + 0.35 M ethanol	59	—	40
2.6×10^{-6} M Catalase + 0.09 M ethanol	—	99	—

* No inhibition by these additions, in the absence of BAL or notatin glucose

peting with X (this X may not be the same substance as X in Scheme *B*) for the H_2O_2 , gave a definite protection. The addition of catalase alone caused a considerable increase in the inhibition, indicating that the reaction $H_2O_2 + X \rightarrow XO$ is catalysed by catalase, when the latter is added in sufficient concentration. The addition of ethanol as well as catalase reduced the inhibition in the presence of catalase alone by about the same proportion as the reduction of the inhibition by ethanol in the absence of catalase. Neither ethanol nor catalase, nor the two together, had these effects on the inhibition caused by BAL. Thus ethanol, which protected from the notatin-glucose inhibition, slightly increased the BAL inhibition, and catalase, which considerably increased the former, had no effect on the latter. This is strong evidence that BAL does not behave as in Scheme *A*. The finding is, however, readily understandable if Scheme *B* correctly formulates the reactions involved. The ethanol added in the presence of catalase reacts with some of the H_2O_2 produced by the copper catalysed oxidation of BAL. This has the effect, however, only of reducing the extent of the other reactions of H_2O_2 (oxidation of BAL, catalytic destruction), without appreciably affecting the relative amounts of BAL oxidized by the two main pathways suggested, and therefore without appreciably affecting the destruction of X .

(3) *Effect of pyruvate on the inhibition of the succinic oxidase system produced by notatin-glucose and by BAL.* It is known from the work of Holleman (1904) and Negelein & Bromel (1939) that pyruvic acid reacts readily with H_2O_2 , as follows



Accordingly pyruvate would be expected to act in the same way as catalase-ethanol, i.e. to have a protective effect if X is oxidized according to Scheme *A*, and to have no effect if X is oxidized according to Scheme *B*. The figures in Table 9 show that pyruvate

Table 9 *Effect of pyruvate on the inhibition of succinic oxidase system produced by notatin-glucose and by BAL*

Pyruvate added System causing inhibition	Inhibition (%)		
	Nil	0.02M	0.1M
0.0031M BAL, 20 min.	53	—	45
0.0094M BAL, 30 min.	98	96	93
Notatin (25 µg) + glucose (10 mg), 30 min	37	—	16

gave considerable protection to the succinic oxidase system against notatin-glucose, but had very little effect on the BAL inhibition, which confirms the conclusions arrived at above. This finding also explains the very small inhibition of succinic oxidase

caused by the D-amino acid oxidase system, since the α -keto acid produced by the oxidation of the amino acid will react with the H_2O_2 also formed, by a reaction similar to that with pyruvate.

Thus it can be concluded that, although H_2O_2 produced by notatin and glucose does cause some inhibition of the succinic oxidase system, the mechanism of this inhibition differs in several respects from that obtained by treatment with BAL. These observations are to be expected from Scheme *B*, but cannot be easily explained by Scheme *A*. There is, however, one possibility which must be considered. Since it is postulated that X is involved in the succinic oxidase system, it is probable that it exists in both an oxidized and a reduced form. In the experiments with BAL, X would be in the reduced form, while in those with notatin-glucose it would be in the oxidized form, and it is possible that reduced X is more susceptible to H_2O_2 than is oxidized X . This explanation was excluded by the experiment which will now be described.

(4) *Effect of cupric ions on the inactivation caused by BAL.* Although copper itself inhibited the succinic oxidase system, this was entirely prevented by BAL, so that the effect of added copper on the inhibition caused by BAL could be studied without any complications arising from the direct effect of copper. Under the conditions of the experiment described in Table 10, $7.2 \times 10^{-5}M$ Cu^{++} trebled the rate of

Table 10 *Effect of cupric ions on the inhibition of the succinic oxidase system produced by BAL*

(Heart-muscle preparation (2 ml), total volume 2.8 ml. Reaction at 39° with shaking in presence of air, for 15 min., 7.2 ml 0.18M phosphate buffer added and 0.2 ml samples used for measurements of enzymic activities.)

	Inhibition (%)	
	Succinic oxidase	Succinic dehydrogenase
0.013M BAL	91	13
0.013M BAL, $7.2 \times 10^{-5}M$ Cu^{++}	66	41

oxidation of BAL in the presence of heart muscle preparation. The presence of Cu^{++} in this concentration gave considerable protection to the succinic oxidase, while the inhibition of succinic dehydrogenase was increased. This extra inhibition of succinic dehydrogenase is probably partly due to the increased rate of production of H_2O_2 and partly due to the increased rate of production of oxidized BAL. The type of inhibition obtained by treatment with BAL in the presence of Cu^{++} somewhat resembles that obtained by treatment with notatin-glucose. It was found in another experiment, not shown in Table 10, that the addition of pyruvate gave some further protection of the succinic oxidase system in the presence of BAL and Cu^{++} (cf. Table 9).

These results would be expected if Scheme *B* correctly describes the reactions, since the addition of copper would promote the copper-catalysed reaction and spare *X*. They cannot be explained by Scheme *A* even if it is assumed that only reduced *X* is susceptible to H_2O_2 , in fact, according to this scheme increased inhibitions might be expected in the presence of Cu^{++} .

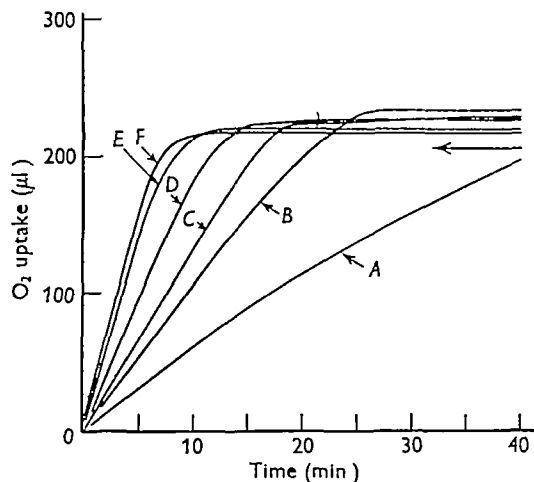


Fig 4 O_2 uptake of BAL + heart-muscle preparation in the presence of varying amounts of cytochrome *c*. All flasks contained 1 ml heart-muscle preparation in a total volume of 2 ml BAL, 0.0094M, temp 37° , pH 7.1. *A*, no addition; *B*, 1.7×10^{-5} M cytochrome *c*; *C*, 3.3×10^{-5} M cytochrome *c*; *D*, 6.6×10^{-5} M cytochrome *c*; *E*, 13.3×10^{-5} M cytochrome *c*; *F*, 16.6×10^{-5} M cytochrome *c*. The horizontal arrow indicates the theoretical O_2 uptake for the reaction $2BAL + O_2 \rightarrow BAL O_2 + 2H_2O$.

It can be concluded that the inhibition produced by BAL cannot be only, or even mainly, due to the H_2O_2 produced by the oxidation of BAL, but must be caused by some other reaction, probably by the directly coupled oxidation (not involving free H_2O_2) of BAL with some substance or grouping necessary for the activity of the succinic oxidase system.

(5) *The effect of cytochrome c on the inhibition of the succinic oxidase system produced by BAL.* As already explained, the rate of reduction of the cytochrome *c* present in the heart muscle preparation by the BAL is insufficient to allow an appreciable rate of oxidation of the BAL through the cytochrome oxidase system, despite the great activity of the latter. Added cytochrome *c* can, however, be rapidly reduced by the BAL and oxidized by the cytochrome oxidase. Thus, the rate of oxidation of the BAL was greatly increased by the addition of cytochrome *c*, the effect of increasing amounts of which is shown in Figs 4 and 5. The initial rate of O_2 uptake in the presence of 16.6×10^{-5} M cytochrome *c* is about 5 times the rate in the absence of additional cytochrome *c*.

The addition of cytochrome *c* provides a third pathway for the oxidation of BAL, additional to those shown in Scheme *B*. Since H_2O_2 is not formed during the oxidation of BAL by the cytochrome *c* cytochrome oxidase system, the oxidation of BAL by this pathway will lead to the theoretical O_2 uptake for the reaction $BAL + \frac{1}{2}O_2 \rightarrow BAL O_2 + H_2O$. Figs 4 and 5 show, in fact, that with increasing cytochrome *c* concentration, the total O_2 uptake decreases towards this theoretical figure. It would

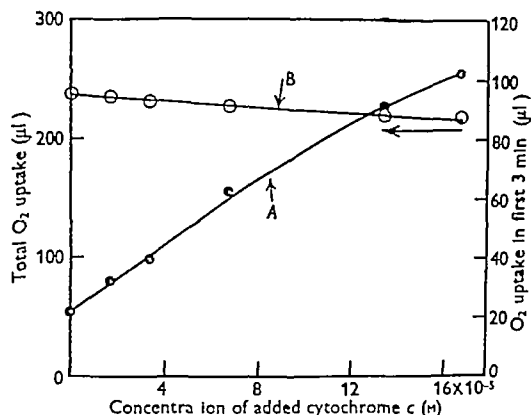


Fig 5 Effect of concentration of cytochrome *c* on initial rate of oxidation of BAL (uptake of O_2 in first 3 min) and on total uptake. Experimental conditions as in Fig 4. *A*, uptake in first 3 min; *B*, total uptake. The horizontal arrow indicates theoretical O_2 uptake for the reaction $2BAL + O_2 \rightarrow BAL O_2 + 2H_2O$.

be expected, from these considerations, that the addition of cytochrome *c*, by providing an additional pathway, not involving *X*, for the oxidation of BAL, would protect *X* from destruction. This prediction is fulfilled by the figures in Table 11, which show that

Table 11 *Effect of added cytochrome c on the inhibition by BAL of the succinic oxidase system*

('General procedure', see p. 15)

Cytochrome <i>c</i> added ($M \times 10^{-5}$)	Percentage inhibition by		
	0.0031M BAL (20 min)		0.0094M BAL (30 min)
	Succinic oxidase (%)	Succinic dehydrogenase (%)	Succinic oxidase (%)
None	53	13	99
3.3	37	16	—
6.6	19	—	65
16.6	0	4	—

the addition of sufficient cytochrome *c* gave complete protection of the succinic oxidase system against the action of BAL. It should be mentioned at this stage that cytochrome *c* does not reverse the inactivation caused by BAL when it is added after the treatment

with BAL. The fact that cytochrome *c* also prevented the small inhibition of the succinic dehydrogenase occurring under the conditions of the experiment described in Table 11 suggests that the inhibition of succinic dehydrogenase is caused not only by oxidized BAL, but also partly by the H_2O_2 produced by the side reaction in Scheme B

Effect of BAL on the cytochrome oxidase activity

Webb & van Heyningen (1947) showed that treatment with 0.005*M* BAL for 15 min at 37° did not affect the cytochrome oxidase activity, measured by the rate of oxidation of catechol in the presence of excess cytochrome *c*. Since it has been shown elsewhere (Slater, 1949*c*) that catechol is not a suitable reducing agent for this measurement, it was considered necessary to re-investigate the effect of BAL, under the conditions used in the present study, on the cytochrome oxidase activity of heart muscle preparation. The cytochrome oxidase activity was measured in 0.15*M* phosphate, the same concentration as used for the determination of the succinic oxidase activity, although this is not optimal for cytochrome oxidase activity.

Table 12 Effect of treatment with BAL on the cytochrome oxidase activity of heart-muscle preparation ('General procedure', see p. 15, 0.0094*M* BAL 30 min.)

Reducing agent	Reducing agent concentration (<i>M</i>)	Phosphate concentration (<i>M</i>)	Concentration of added cytochrome <i>c</i> (<i>M</i> × 10 ⁵)	Inhibition of O ₂ uptake (%)
Quinol	0.05	0.15	6	11
Ascorbic acid	0.025	0.15	2	13
	0.025	0.15	4	11
	0.025	0.15	6	13
<i>p</i> Phenylene diamine	0.05	0.15	0	32
	0.05	0.15	1	26
	0.05	0.15	3	19
	0.05	0.15	6	16
	0.05	0.007	6	3
	0.05	0.089	6	10
	0.05	0.144	6	17
	0.05	0.401	6	25
	0.05	0.007	0	20
	0.05	0.089	0	24
	0.05	0.144	0	36
	0.05	0.401	0	18
	0.003	0.15	0	22
	0.0075	0.15	0	31
	0.02	0.15	0	39
	0.05	0.15	0	42

Treatment of either heart muscle or kidney preparations with BAL caused very little inhibition of the rate of oxidation of quinol or of ascorbic acid in the presence of the enzyme and cytochrome *c*, and this small inhibition was not affected by the con-

centration of added cytochrome *c* (Table 12). However, when *p* phenylenediamine was the reducing agent and heart muscle the source of the enzyme, there was a considerable inhibition of the rate of oxidation in the absence of cytochrome *c*, and, although this was decreased by the addition of cytochrome *c*, it was still considerable in the presence of 6×10^{-5} *M* added cytochrome *c*.

The figures for *p* phenylenediamine are given graphically in Fig. 6. In Fig. 6*b*, the two lines cross the ordinate at the same point, i.e. at infinite cytochrome *c* concentration the BAL treated heart muscle preparation has the same cytochrome oxidase

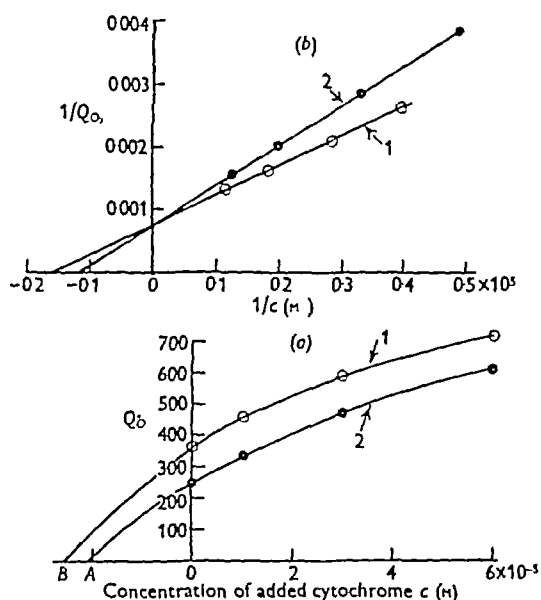


Fig. 6 (a) Effect of treatment with BAL on the capacity of heart-muscle preparation to catalyse the oxidation of *p* phenylenediamine. Heart-muscle preparation treated by 'general procedure' (p. 15) for 30 min at 38°, with shaking in the presence of air, *p* phenylenediamine (0.05*M*), phosphate buffer (0.15*M*). 1, control; 2, treated with BAL (0.0094*M*). The curves have been extrapolated to the abscissa by assuming that the points fall on a rectangular hyperbola.

(b) Figures in (a) plotted according to the procedure of Lineweaver & Burk (1934). The distances *OA* and *OB*, found in (a), have been added to the concentration of added cytochrome *c*.

activity as the control. Treatment with BAL has, however, affected the catalytic activity of the added cytochrome *c*; thus if (*cyt c*)₁ is the concentration of cytochrome *c* required for half maximal activity (Slater, 1949*c*), $1/(\text{cyt } c)_1$ for the BAL treated preparation is $0.12 \times 10^5 \text{ M}^{-1}$, compared with $0.165 \times 10^5 \text{ M}^{-1}$ for the control. It has also affected the catalytic activity of the endogenous cytochrome *c*. Thus the extrapolation in Fig. 6*a* shows that the

untreated preparation contains cytochrome *c* equivalent to 2.52×10^{-5} M added cytochrome *c*, while the endogenous cytochrome *c* of the BAL treated preparation is equivalent to 2.05×10^{-5} M added cytochrome *c*, but the added cytochrome *c* is only 0.12/0.165 times as effective in the latter case compared with the control, i.e. the endogenous cytochrome *c* of the BAL treated preparation is $2.05/2.52 \times 0.12/0.165 \times 100 = 59\%$ as effective as that of the untreated preparation. The actual amount of cytochrome *c* is unaffected by this treatment (see below).

The finding that BAL affects not the cytochrome oxidase of the heart muscle preparation, but the catalytic activity of the added and the endogenous cytochrome *c* explains other conclusions drawn from Table 12 and additional experiments, viz —

(1) The degree of inhibition was decreased by lowering the concentration of enzyme while the concentration of added cytochrome *c* remained constant. Lowering the concentration of enzyme decreases the proportion of the oxidation proceeding through the endogenous cytochrome *c*, since the amount of added cytochrome *c* approaches more closely to that required for maximum activity of the cytochrome oxidase (Slater, 1949c).

(2) The degree of inhibition was decreased by lowering the phosphate concentration, which has the same effect as reducing the enzyme concentration (Slater, 1949c).

The catalytic activity of the endogenous cytochrome *c* is determined by two factors, viz (1) the accessibility of *p*-phenylenediamine to this cytochrome *c*, and (2) the accessibility of the cytochrome *c* to the oxidase on the particles of the enzyme preparation. The finding (Table 12) that the degree of inhibition in the absence of added cytochrome *c* was decreased by lowering the concentration of reducing agent shows that it is (2) which is affected by the treatment with BAL. At low *p*-phenylene diamine concentrations, the rate of reduction of cytochrome *c* becomes the limiting factor in both the control and BAL treated preparations, so that the impaired rate of oxidation of the cytochrome *c* caused by the treatment with the BAL is masked.

Since the effect of BAL on the rate of oxidation of *p*-phenylenediamine is due, primarily, to an effect on the accessibility of the endogenous cytochrome *c* of the heart muscle preparation to the cytochrome oxidase, it is not surprising that the oxidation of ascorbic acid and quinol are little affected, since the endogenous cytochrome *c* plays only a small part in the oxidation of these substances. The inhibition of the oxidation of *p*-phenylenediamine disappears when sufficient cytochrome *c* is added, but it is interesting to find that the catalytic activity of the added cytochrome *c* is also affected by treatment with BAL. Since this was not the case when other

reducing agents were used, it seems that this is due to an effect on the rate of reduction of the added cytochrome *c* (in the vicinity of the oxidase) by *p*-phenylenediamine. This is probably because the *p*-phenylenediamine cannot approach so closely to the cytochrome oxidase after treatment of the heart muscle preparation with BAL.

Thus the effect of treatment of the heart muscle preparation with BAL on the rate of oxidation of *p*-phenylenediamine is due to an impairment of the accessibility, both of the endogenous cytochrome *c* and of the *p*-phenylenediamine, to the cytochrome oxidase.

Treatment of the heart-muscle preparation with BAL in the absence of air had no effect on the power of heart muscle preparation to catalyse the oxidation of *p*-phenylenediamine.

Identification of the BAL sensitive component of the succinic oxidase system

(1) *Spectroscopic observations* No bands were observed when a heart muscle preparation, treated with 0.016 M BAL for 30 min at 37° with shaking in air, was placed in a test tube under a microspectroscope. After adding succinate and allowing to stand, the bands of reduced cytochromes *a* + *a*₃, *b* and *c* all appeared. When the mixture was then shaken for a few seconds, the *b* band remained visible, but the *a* + *a*₃ and *c* bands disappeared and did not return until the mixture had been standing without shaking for about 10 min. Thus, in the BAL-treated heart muscle preparation, the reduction of cytochrome *b* and the oxidation of cytochromes *c* and *a* + *a*₃ were not affected, but the oxidation of cytochrome *b* and the reduction of cytochromes *a* + *a*₃ and *c* were greatly impaired. The cytochrome *b* could, however, be readily oxidized by methylene blue or by 2,6-dichlorophenolindophenol.

The positions and intensities of the three bands of the reduced cytochromes after the addition of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) were not affected by treatment with BAL, but the total protohaematin content of the heart muscle preparation, measured by the intensity of the pyridine haemochromogen band at 550–560 mμ, was reduced by about 20% by treatment with BAL (Table 13). It follows that some haematin compound, the spectrum of which is not visible in the heart muscle preparation (Slater, 1949a), is destroyed by the treatment with BAL. The amount of haematin compound destroyed is about 30% of the difference between the cytochrome *b* and total protohaematin contents, as calculated in the latter paper. It is known that BAL has a destructive effect on some haematin compounds. Thus, Barron, Miller & Kalnitsky (1947) have shown that haemoglobin is destroyed by treatment with BAL in the presence of air, and, in the present investigation, it was found that myoglobin was similarly

affected This reaction is probably essentially the same as that between haemoglobin and another reducing agent, ascorbic acid, which has been studied in detail by Lemberg, Legge & Lockwood (1941) If this is the case, the product of the coupled oxidation of the haematin compound with the BAL would be related to the bile pigments These pigments have only weak absorption bands, and, considering that the spectrum of the haematin compound is itself not visible, it is not surprising that no such bile pigment could be detected spectroscopically

Treatment with $\text{Na}_2\text{S}_2\text{O}_4$ and pyridine shifts the $a+a_3$ band from 603–607 μ to 580–590 μ , thus

and the inhibition of the succinic oxidase system This is most clearly seen in Exp 3, in which different concentrations of BAL were employed Under the conditions of Exp 2, the BAL was completely oxidized in 25 min The fact that the reduction in intensity of the band was about the same after 60 min treatment as after 10 min shows that oxidized BAL is not responsible for the destruction of the haematin compound This is supported by the finding that another disulphide, oxidized glutathione, had no appreciable effect on the haematin content BAL under anaerobic conditions did not cause any destruction of the haematin compound or inhibition

Table 13 *Destruction of haematin compound in heart muscle preparation by treatment with BAL under various conditions and with oxidized glutathione and notatin glucose*

(Destruction measured by decrease of intensity of 550–560 μ band produced by adding $\text{Na}_2\text{S}_2\text{O}_4$ and pyridine to heart muscle preparation, 2 ml heart-muscle preparation in total volume 2.4–2.8 ml Aerobic treatment in all experiments except for one marked*)

Exp no	Heart muscle preparation no	Treatment		Destruction of haematin compound (% of total)	Inactivation of succinic oxidase system (%)
		Reagent	Time (min)		
1	20	0.0085M BAL	15	42	75
2	21	0.0085M BAL	10	16	—
		0.0085M BAL	20	21	—
		0.0085M BAL	30	23	—
		0.0085M BAL	60	18	—
		0.0039M BAL	15	6	37
3	21	0.0077M BAL	15	13	73
		0.0155M BAL	15	15	98
		0.031M BAL	15	21	99
		0.013M BAL	15	16	92
4	22	0.013M BAL	15*	1	0
		0.1M oxidized glutathione	15	0	47†
5	22	50 μ g notatin, 10 mg glucose	30	0	12
		0.013M BAL	15	24	100
6	23	0.013M BAL	15	24	100
7	27	0.0157M BAL	15	14	100
8	29	0.0157M BAL	15	9	100

* Anaerobic treatment

† Inhibition of succinic dehydrogenase, 34%

latter band is considerably weaker than the band at 548–560 μ , and, in view of its proximity to the latter, accurate measurements of its intensity were difficult It is not known, therefore, whether treatment with BAL leads to any destruction of haematin compounds which yield a pyridine haemochromogen with an absorption band in this position, i.e. those which contain the haem of cytochromes $a+a_3$, but there was no weakening of the $a+a_3$ band obtained with $\text{Na}_2\text{S}_2\text{O}_4$ alone, which is consistent with the finding that the cytochrome oxidase activity was not affected

The results in Table 13 show that the amount of BAL labile haematin compound varies from preparation to preparation, but is usually about 14–24% The figures further show some correlation between the degree of the reduction of intensity of the band

of the succinic oxidase system Notatin glucose had no measurable effect on the haematin compound

There was no detectable destruction of the 548–560 μ band in the case of the kidney preparation However, if the BAL labile haematin compound was present in this preparation in about the same concentration relative to that in heart muscle, as are the cytochromes, this compound would be such a small proportion of the total protohaematin content that its destruction would be hardly detected

(2) *Examination of possibility that the inactivation caused by BAL is due to purely physical factors* Two possible explanations of the effect of BAL on the succinic oxidase system may be considered (1) Treatment with BAL may so change the structure of the particles of the heart muscle preparation that, for purely physical reasons, the cytochrome b is no

longer accessible to the remainder of the system, but can be oxidized by the small molecule methylene blue (2) The substance destroyed by the BAL is actually a component of the succinic oxidase system, required to transmit electrons from cytochrome *b* to cytochrome *c*

The nature of the inactivation of the succinic oxidase system, which has all the characteristics of an actual destruction of a substance, makes the second explanation more likely In another paper (Slater, 1949*b*), it is shown that inhibitors of the first type, i.e. those which act non specifically on the enzyme system by affecting the particles of the enzyme preparation, possess certain characteristics which enable them to be distinguished from the more specific inhibitors The non specific inhibitors (1) usually inhibit the cytochrome oxidase as well as the succinic oxidase system, (2) affect the succinic oxidase system to a greater extent if cytochrome *c* is not added during the measurement of the enzyme activity, and (3) the inhibition is often reversed by calcium phosphate gel or denatured proteins The inhibition of the succinic oxidase system by BAL possesses none of these characteristics The cytochrome oxidase activity is hardly affected, denatured globin, serum proteins or calcium phosphate gel do not reactivate the system after complete inhibition by the BAL and cytochrome *c* does not affect the inhibition, even in the presence of denatured globin (Table 14) It is,

oxidase is impaired after treatment with BAL, is inconsistent with this conclusion However, the effect on the accessibility of cytochrome *c* to the oxidase is small compared with the complete inactivation of the succinic oxidase system, and is probably secondary to it The destruction of the factor necessary for the reduction of cytochrome *c* by cytochrome *b* would make this step in the reaction sequence the rate determining reaction, so that addition of cytochrome *c*, although it could overcome the minor effect on the oxidation of cytochrome *c*, would have no effect on the degree of inhibition of the succinic oxidase activity

Other attempts to reactivate the BAL inactivated system

In addition to the substances already mentioned (*viz* denatured globin, serum proteins, calcium phosphate gel and cytochrome *c*), the following were found to be unable to reactivate the enzyme system after treatment with BAL catalase, Straub's 'SC factor' (Straub, 1942, Keilin & Hartree, 1949, Slater, 1949*b*), aqueous extract of minced horse heart (rich in myoglobin), supernatant from the isoelectric precipitation of heart muscle extract in phosphate buffer, and heart muscle preparation treated with sufficient *p* aminophenylarsenoxide to inhibit completely the succinic dehydrogenase activity It is shown elsewhere (Slater, 1949*d*) that *p* aminophenylarsenoxide inhibits the succinic oxidase system by

Table 14 *Effect of cytochrome c (added after BAL treatment) on the degree of inactivation of the succinic oxidase system produced by BAL*

(Exp 1-3 heart-muscle preparation, treated by 'general procedure', with 0.003*M* BAL for 20 min Exp 4 kidney preparation, treated by 'General procedure', with 0.0015*M* BAL for 15 min)

Exp no	Phosphate concentration (M)	Globin concentration (%)	Cytochrome <i>c</i> concentration ($\mu\text{M} \times 10^5$)	Succinic oxidase activity ($\mu\text{L}/10 \text{ min}$)		Inhibition (%)
				Control	BAL-treated	
1	0.14	0	0	90	43	52
	0.14	0	4	116	57	51
	0.14	0.13	0	100	59	41
	0.14	0.13	4	132	80	40
2	0.15	0	0	50	18	63
	0.15	0	4	83	30	64
3	0.03	0	0	23.8	3.2	87
	0.03	0	4	36.2	5.4	85
4	0.10	0	0	8.7	4.0	55
	0.10	0	1	19.1	7.6	60
	0.10	0	2	22.2	10.0	55
	0.10	0	6	29.3	8.3	72

therefore, concluded that the second explanation, *viz* that BAL destroys a component of the succinic oxidase system required to transmit electrons from cytochrome *b* to cytochrome *c*, is the correct one

It might appear at first sight that the finding, from experiments with *p* phenylenediamine, that the accessibility of the endogenous cytochrome *c* to the

combining with the succinic dehydrogenase, thus, heart muscle preparation treated with the arsenical will probably still contain the factor linking cytochrome *b* with cytochrome *c* It is not surprising, however, that a heart muscle preparation treated with this arsenical was unable to reactivate the enzyme system after treatment with BAL since the

inactive system, lacking the factor but containing the succinic dehydrogenase, and the inactive system which has this factor intact but whose succinic dehydrogenase is inhibited, would be on different particles and would be quite inaccessible to one another

DISCUSSION

There are four possible pathways for the oxidation of BAL in the presence of heart muscle preparation, viz by (1) Cu^{++} ions introduced during the preparation or by the buffer solution, (2) copper protein compounds, (3) cytochrome *c* and oxidase, (4) the BAL-labile factor. The actual proportion of the total oxidation passing through the BAL labile factor, which is the only pathway leading to the destruction of this factor, is not known, but it may be quite small which would explain why relatively large concentrations of BAL are necessary to inactivate the succinic oxidase system

transfer of electrons from cytochrome *b* to cytochrome *c*

The relationships between this factor and the cytochromes are shown in Fig 7, the arrows showing the direction of electron transfer. It can be seen from this diagram that the destruction of the factor would not affect the oxidation of succinate through methylene blue as carrier, nor the oxidation of various reducing agents through cytochrome *c* and cytochrome oxidase, but would completely prevent the transfer of electrons from cytochrome *b* to cytochrome *c* and thence to molecular oxygen. It has been shown previously (Slater, 1948) that treatment with BAL under conditions which caused complete inactivation of the succinic oxidase system, with about 10% inhibition of succinic dehydrogenase, inhibited the activity of the system, measured anaerobically by the rate of reduction of potassium ferricyanide, by 35%. This is probably because potassium ferricyanide, with an oxidation reduction

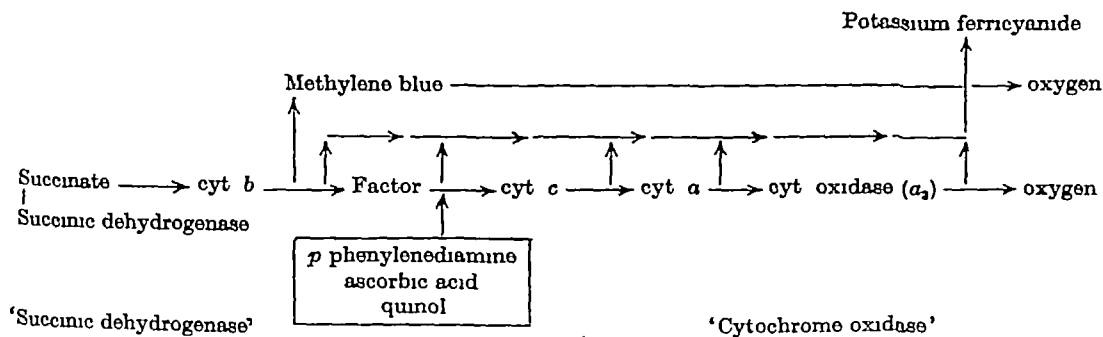


Fig 7 The components of the succinic oxidase system

Of the reducing agents mentioned in Table 1, only BAL has been studied intensively. It seems probable however, that the mechanism of the action of the other SH compounds (except diethyldithiocarbamate) and of ascorbic acid on the succinic oxidase system is similar to that of BAL. The larger inhibition of the succinic dehydrogenase found with ascorbic acid compared with BAL may be due to the failure of ascorbic acid ($E'_0 = 0.05 \text{ V}$, at pH 7.3, 30°, Ball, 1937) to protect the SH groups of succinic dehydrogenase from oxidation by H_2O_2 . BAL with an E'_0 of -0.15 V at pH 7.0 (Barron, Miller & Kalnitsky, 1947) is more favourably situated in this respect. The greater inhibition with cysteine is probably related to the more rapid oxidation of this compound which might be expected to behave in the same way as BAL, if a shorter time of reaction were used.

The chief finding of this investigation is that the primary effect of BAL on the succinic oxidase system is due to the destruction, by directly coupled oxidation, of a factor which is necessary for the

potential ($E'_0 = 0.41 \text{ V}$, at pH 7.3) much higher than that of succinate \rightleftharpoons fumarate ($E'_0 = -0.01 \text{ V}$, at pH 7.3) or of cytochrome *b* ($E'_0 = -0.04 \text{ V}$, at pH 7.3), operates in part directly with cytochrome *b* or succinate (activated by the dehydrogenase), and in part through the BAL labile factor or the cytochromes of higher oxidation reduction potential (E'_0 of cytochrome *c* = $+0.26 \text{ V}$, of cytochrome *a* = $+0.29 \text{ V}$). Thus the destruction of the factor causes a partial, but not complete, inhibition of the rate of oxidation of succinate by ferricyanide.

It seems likely that this factor is a haematin compound. The evidence for this is (1) the inactivation, which occurs only when the reducing agent is added to the enzyme preparation in the presence of air, bears a striking resemblance to the effect of reducing agents on haemoglobin (Lemberg *et al.* 1941), (2) BAL has been shown to destroy haematin itself and haemoglobin (Barron, Miller & Kalnitsky, 1947), myoglobin (this investigation) and the haematin enzymes peroxidase and catalase (Webb & van Heyningen, 1947, Lemberg & Foulkes, 1948),

while glutathione inhibits catalase (Marks, 1936), (3) quantitative measurements of the amount of haematin compounds in the heart muscle and kidney preparations suggest that these preparations contain haematin compounds, whose spectra are not visible (Slater, 1949*a*), (4) some protohaematin compound is destroyed by treatment of the heart muscle preparation with BAL, and the destruction of this compound is parallel to the inactivation of the succinic oxidase system

It is interesting to note that the naturally occurring haematin compounds fall into two groups, according to the nature of their absorption spectra and their susceptibility to reducing agents, viz —

Group A peroxidase, catalase and methaemoglobin, these compounds, whether in the ferric or the ferrous state, possess rather weak absorption bands. They are destroyed by reducing agents in the presence of air

Group B cytochromes *b*, *c*, *a*, *a*₃, these compounds possess haemochromogen spectra, i.e. strong bands in the ferrous state, weak bands in the ferric state. They are not affected by reducing agents

According to this classification, the factor in the succinic oxidase system belongs to Group A

Keilm & Hartree (1949) have suggested that this factor might be related to cytochrome *c*₁, which, according to Yakushiji & Okuniki (1940), acts between cytochromes *b* and *c* in the succinic oxidase system. However, it has been shown in another study (Slater, 1949*e*) that the evidence for the existence of cytochrome *c*₁ is unsatisfactory

The possibility should be considered that the action of the BAL is not on an additional factor, but on cytochrome *b* itself. This could be the case, only if the BAL affects the cytochrome *b* in such a way that its absorption spectrum is unaltered and its reduction is unaffected, but it can no longer be oxidized. This possibility, itself extremely unlikely, is disproved, first, by the evidence that cytochrome *b* is a part of, if not identical with, succinic dehydrogenase (see Slater, 1949*a*) and, secondly, by the observation that, after the treatment with BAL, the cytochrome *b* could still be readily oxidized by dyestuffs

A consideration of the oxidation reduction potentials of the known hydrogen and electron carriers reveals that there is a big gap in potential between cytochrome *b* and *c*. The finding of a factor which operates between these two cytochromes narrows this gap. This factor does not account for all the protohaematin found in tissue preparations, and it is possible that some other haematin compound is also involved in the succinic oxidase system

The question must now be considered whether the factor operates only in the succinic oxidase system or is a catalyst in the main pathway of respiratory catalysis. If cytochrome *b* is a catalyst in the main pathway, it follows that the factor must be a

respiratory catalyst concerned in the oxidation of most substrates. If, however, as is possible, cytochrome *b* is concerned only in the oxidation of succinate, the factor may either be in the main pathway between diaphorase and cytochrome *c*, or, like cytochrome *b*, be concerned only with the oxidation of succinate and not with that of those substrates which act through the nicotinamide nucleotides and diaphorase. It is known that pure cytochrome *c* cannot be reduced by pure diaphorase (Lockhart & Potter, 1941). It is not unlikely that the factor required for the reduction of cytochrome *c* by cytochrome *b* is also required, either alone or in conjunction with cytochrome *b*, for the reduction of cytochrome *c* by diaphorase. This can only be decided by further experiments, which are in progress

Another dehydrogenase which, like succinic dehydrogenase, reacts with the cytochromes without the intervention of the nicotinamide nucleotides or diaphorase, is the lactic dehydrogenase of yeast which is probably identical with cytochrome *b*₂, a haematin compound, whose spectrum is not visible in yeast, but which was obtained in a concentrated form by Bach *et al* (1946). These authors showed that the rapid reduction of cytochrome *c* by cytochrome *b*₂ required an additional factor (*X*), which is removed in the purification and which is not required for the reduction of methylene blue. It is possible that this factor (*X*) is the same as, or closely related to, the factor under consideration. There is, indeed, some evidence in the literature that yeast contains a respiratory catalyst, which is sensitive to thiols (Quastel & Wheatley, 1932, Runnstrom & Sperber, 1938, Cook & Persutti, 1947)

p Phenylenediamine differs from the other reducing agents used in the estimation of cytochrome oxidase activity, first, by being more accessible to the endogenous cytochrome *c* of the enzyme preparation, and, secondly, by being more accessible to added cytochrome *c* in the vicinity of the oxidase (Slater, 1949*c*). The latter, but probably not the former, is affected by treatment with BAL. The reason for this difference in behaviour of *p* phenylenediamine is unknown. Since treatment with BAL destroys a factor necessary for the reduction of cytochrome *c* by cytochrome *b*, the possibility that this factor catalyses the reduction of cytochrome *c* by *p* phenylenediamine should be considered. However, the following evidence is strongly against this view: (1) the main reason for the inhibition of the *p* phenylenediamine oxidation is not an inhibition of the rate of reduction of cytochrome *c* by *p* phenylenediamine, but an effect on the accessibility of this cytochrome *c* to the oxidase (i.e. on its rate of oxidation), (2) treatment with BAL under conditions which completely inactivated the succinic oxidase system inhibited the rate of oxidation of *p* phenylenediamine by only 30–40 %

Several workers (see Table 15) have reported inhibitors which affect the complete succinic oxidase system much more markedly than the succinic dehydrogenase and cytochrome oxidase portions. These inhibitors may be of two types, viz (1) those which react specifically with the factor which links the succinic dehydrogenase to the cytochrome oxidase, and (2) those which act non specifically on the enzyme system, by affecting the mutual accessibility of the components, on the particles of the enzyme preparation.

Table 15 lists these inhibitors, together with their probable mode of action, which has been investigated thoroughly in the case of only the first three in-

active chemically, but is strongly adsorbed on protein films (Rideal & Shulman, 1939). It is clear that further work is required to elucidate the mechanism of the action of these interesting new inhibitors introduced by Ball *et al* (1947) and Case & Dickens (1948). Their findings reported to date, like those of Hopkins *et al* (1939), Stern & Melnick (1939), Straub (1942), and Stoppani (1947) (see p 14, and Keilin & Hartree, 1949) do not, in themselves, provide any evidence for the existence of a factor between the dehydrogenase and cytochrome oxidase. The reducing agents (in the presence of air) studied in the present paper remain as the only substances known at present to inactivate the factor

Table 15 *Inhibitors of succinic oxidase system which inhibit complete system more than succinic dehydrogenase or the cytochrome oxidase*

Inhibitor	Reference	Probable mechanism of action
Bile salts	Straub (1942), Keilin & Hartree (1949), Slater (1949b)	Non specific
Laser's haemolytic substance (Laser & Friedmann, 1945)	Slater (1949b)	Non specific
Reducing agents + oxygen	Present paper	Destruction of component
Oxidizing agents	Slater (1949d)	Destruction of component (?)
p Chloromercuribenzoate	Slater (1949d)	Non specific (?)
Fluoride	Borel (1945)	?
Pyocyanine	Keilin & Hartree (1940)	?
2-Hydroxy 3 alkyl 1 4-naphthaquinones	Ball <i>et al</i> (1947)	?
4 4' Dihydroxystilbene	Case & Dickens (1948)	?

hibitors. In another paper (Slater, 1949d), reasons are given for believing that oxidizing agents and p chloromercuribenzoate act in the manner described, these inhibitors have not, however, been studied in detail. It is impossible to decide, on the basis of the available evidence, the mode of action of fluoride or pyocyanine. It is quite possible that fluoride combines with the BAL labile factor, with which the present paper is concerned, since there is evidence that this factor is a haematin compound and many haematin compounds combine with fluoride.

Both Ball *et al* (1947) and Case & Dickens (1948) believe that the compounds which they studied acted on a factor linking the succinic dehydrogenase with the cytochrome oxidase, but the alternative hypothesis has not been investigated by these workers. Indeed, there is reason to believe that the naphthaquinones might act non specifically, since only hydroxynaphthaquinones with long aliphatic side chains were effective inhibitors. The length of the aliphatic side chain could not appreciably affect the chemical properties of a naphthaquinone, but would profoundly affect its physical properties. The fact that succinic dehydrogenase was inhibited to a certain extent by these compounds is also in agreement with this interpretation (cf Slater, 1949b). Similarly, 4 4' dihydroxystilbene, the compound studied by Case & Dickens (1948), is not very re-

SUMMARY

1 The succinic oxidase system of heart muscle and kidney preparations is inactivated by treatment with a number of reducing agents in the presence of air. Under certain conditions, many of these reducing agents have little effect on the succinic dehydrogenase.

2 BAL has two quite distinct effects: (a) complete inactivation of the succinic oxidase system, without any effect on the succinic dehydrogenase, (b) a partial inhibition of succinic dehydrogenase, which does not occur until more than half the BAL has been oxidized. The latter inhibition is probably due to oxidation of SH groups. The former inhibition was studied in detail.

3 Oxidized BAL inhibited the succinic oxidase system much less than BAL, while no inhibition occurred if the enzyme preparation was treated with BAL under anaerobic conditions.

4 H_2O_2 is produced during the oxidation of BAL in buffer solution or in the presence of heart muscle preparation. Some substance (or substances) is oxidized during treatment of heart muscle preparation with BAL in the presence of air.

5 H_2O_2 produced by notatin glucose and by D amino acid oxidase and its substrates had a much less pronounced effect on the succinic oxidase system than BAL. Inhibition by these substances was

increased by catalase and decreased by ethanol and pyruvate. Catalase, ethanol and pyruvate had no effect on the inhibition of the system caused by BAL.

6 The addition of copper, which increased the rate of oxidation of BAL, decreased the inactivation of the succinic oxidase system, but increased that of succinic dehydrogenase.

7 It is concluded that the inhibition produced by BAL is not due to the H_2O_2 formed during the oxidation of BAL, but is caused by the directly coupled oxidation of BAL, not involving free H_2O_2 , with some substance or grouping necessary for the activity of the succinic oxidase system.

8 The presence of cytochrome *c*, during the treatment of the enzyme with the BAL, prevented the inactivation of the enzyme.

9 Treatment with BAL does not affect the true cytochrome oxidase activity.

10 Treatment with BAL did not destroy the cytochromes, a protohaematin compound, amounting to about 20 % of the total protohaematin content of the heart muscle preparation, was, however, destroyed by this treatment. There was some correlation

between the amount of haematin compound destroyed and the degree of inactivation of the succinic oxidase system when different concentrations of BAL were allowed to act on the enzyme for various times.

11 Treatment with BAL caused an impairment of the oxidation of cytochrome *b* by cytochrome *c*. The cytochrome *b* could, however, be oxidized by methylene blue or dichlorophenolindophenol.

12 Evidence is presented in favour of the view that BAL affects the succinic oxidase system by destroying a component of the succinic oxidase system required for the transmission of electrons between cytochrome *b* and cytochrome *c*.

13 The component of the succinic oxidase system, which is destroyed by the treatment with the BAL, is probably the same as the haematin compound destroyed by the same treatment.

I would like to express my deep appreciation of the interest taken by Prof. D. Keilin, F.R.S., and for his stimulating suggestions throughout the course of this investigation. I would also like to thank Dr. E. F. Hartree for supplying the catalase and D-amino acid oxidase and for many valuable suggestions, and the British Council for a scholarship.

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Addendum (15 June 1949)

It has now been found (Slater 1949f) that the respiratory catalyst discussed in this paper is also necessary for the oxidation of dihydrocozymase, acting between diaphorase and cytochrome *c* (cytochrome *b* is not required

for this oxidation). Thus, the factor links the succinic oxidase and dihydrocozymase oxidase systems and enables the anaerobic oxidation of dihydrocozymase by fumarate

Nitrogen in Human Dental Enamel

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(Received 7 January 1949)

Dental enamel is the structure which is first exposed to the attack of dental caries, and the success or failure of this attack depends on many factors, among them the chemical nature and structure of the enamel. Some authors (Pincus, 1939, Gottlieb, 1947) go so far as to postulate that the caries process is initially a proteolytic one. In this connexion the nitrogenous material of enamel is of interest.

The object of the present experiments was to estimate the nitrogen content of enamel on samples of material which were representative of the main enamel mass and uncontaminated by dentine. Enamel is firmly attached to the underlying dentine which contains about 3.5% nitrogen compared with about 0.15% in enamel. On these figures, even a 2% dentine impurity would increase the enamel nitrogen by about 60%.

METHOD

The following methods are available for the preparation of enamel samples: (i) The enamel may be ground off with various cutting instruments. With this method, apart from the difficulty in judging when the dentine is about to be exposed, there must always be a variable amount of enamel attached to underlying dentine which is not taken for analysis. (ii) The dentine may be removed from the inside with burs, leaving a hollow enamel cap. (iii) The whole calcified crown of the tooth may be removed and powdered, and the dentine and enamel separated by a flotation method relying on the difference of density between the two materials. This method was used by Manley & Hodge (1939), who claimed for it an enamel purity of 99.4%, but a loss of enamel of 9.3%. Since this fraction is necessarily slightly less dense than the remainder, it is probable that it contains proportionally more organic material, so that the nitrogen loss may be considerably more than 9.3% of the total.

Method (ii) was considered the most reliable. The teeth used in the first part of the investigation were sound premolars which had been extracted for orthodontic purposes from patients not more than 16 yr old. The teeth were collected in 70% ethanol and thoroughly scraped and brushed on a lathe with pumice powder before use. The crowns were then removed and dentine burred out leaving a hollow enamel cap.

With round burs of graded size, dentine was removed rapidly and completely. The matt appearance of dentine, compared with the shiny undersurface of the enamel coupled with the change in burring sound and resistance, made the arrival at the amelodentinal junction easily detectable. There was small risk of removing enamel with these burs as very light pressure was used.

For each batch the enamel from 6 to 10 crowns was crushed in a diamond mortar to a powder which would pass a 60 mesh sieve. The purity of the powder was tested by the method described by Manley & Hodge (1939), relying on the different refractive indices of enamel and dentine. With this method it was possible to recognize under the microscope particles of dentine in a field of enamel particles. Tests were made on every batch and never more than three dentine particles per thousand enamel particles were found. The powder was then divided into three samples of between 500 and 700 mg, dried to constant weight at 105–110° and each sample was transferred to a 100 ml Kjeldahl digestion flask containing 7 ml of HCl (1 vol conc HCl plus 4 vol water) to dissolve the calcified material. Digestion with concentrated H_2SO_4 , using the catalyst of Chibnall, Rees & Williams (1943), was carried out for 11 hr. The NH_3 was steam-distilled into 1% (w/v) boric acid and titrated with 0.01N HCl, using the methyl red-methylene blue indicator mixture of Pierre, Tully & Ashburn (quoted by Britton, 1942).

It was thought that the presence of $CaSO_4$ in the digest might interfere with the recovery of the NH_3 . To test this point estimations were carried out on egg albumin solutions in the absence and presence of $CaSO_4$. The results given below showed that there was no interference. (Sample 1 without $CaSO_4$, 0.119 mg/l, with $CaSO_4$, 0.118 mg/l. Sample 2 without $CaSO_4$, 0.122 mg/l, with $CaSO_4$, 0.122 mg of N/l.)

RESULTS

Table 1 shows the nitrogen content of ten batches of enamel. In all cases except two the values given are the means of estimations on three samples of a batch of enamel obtained by pooling 6–10 crowns. Wide variation is shown about the mean of 0.071 g/100 g.

Table 1. Nitrogen content of dental enamel taken from (a) sound young premolars and (b) sound teeth from patients in higher age groups.

N (g/100 g enamel)	
Orthodontic extractions (under 17 yr of age)	Caries resistant older age groups
0.110	0.071
0.052	0.086
0.078	0.069
0.097	0.102
0.059	0.137
0.072	0.099
0.056	0.062
0.062	0.058
0.062	0.077
0.059	0.065
Mean \pm s D	0.071 \pm 0.015
	0.083 \pm 0.021

enamel, and this is expressed by the standard deviation of ± 0.015 g/100 g

A second series of estimations was conducted on teeth which had resisted caries. The teeth used were sound premolars extracted because of paradontal disease or to clear an otherwise empty mouth, and came from the higher age groups. The values, again the means of three samples, are also shown in Table I. With a mean of 0.083 g/100 g enamel, and standard deviation of ± 0.021 g/100 g there is no statistically significant difference between the nitrogen content of this enamel and that of the first series.

DISCUSSION

The values for enamel nitrogen found in the experiments reported here are lower than most reported to date. Bowes & Murray (1935) found 0.156% in enamel separated by grinding it from the surface of the dentine. Deakins & Volker (1941) used the flotation method for separating enamel and dentine. From their figures expressed as protein with a nitrogen content assumed to be 16% the range of nitrogen values was found to be 0.078–0.31%. It is considered that the low results given in the present paper were obtained by reducing the dentine

impurity to a minimum. A simple comparison between the enamel-nitrogen content of the two groups of teeth gives little information. In this series at least two variants may operate. First, there is the possibility that age changes may influence nitrogen content. Secondly, on the basis of a proteolytic theory of dental caries there is a possible relationship between nitrogen content and caries susceptibility. Although the second series of estimations were conducted on teeth of proven caries resistance, the orthodontic premolars were of unknown susceptibility to caries, some would have survived, others would have succumbed. Further work is necessary to test the effect of these and other variants on enamel nitrogen values.

SUMMARY

Nitrogen estimations carried out on the carefully separated enamel of sound premolar teeth give a mean value of 0.071 ± 0.015 % for the age group up to 16 years and 0.083 ± 0.021 % for older age groups. These values are lower than those previously reported.

My thanks are due to Dr Agnes Shore, Prof W R Spurrell and Prof M A Rushton for much helpful advice and criticism.

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Interrelationship of Certain Vitamins of the B Group in Aneurin, Riboflavin and Biotin Deficiencies

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(Received 1 November 1948)

A close interrelationship of vitamins of the B group may be expected, since they are fundamental to all forms of life, and are the controlling factors in the chain of oxidative removal of certain metabolites. Definite interrelationships among these vitamins have been established by investigations on human subjects and on animals. The treatment of multiple deficiencies in man with aneurin alone results in the development of pellagrous skin changes which disappear on administration of either nicotinic acid or

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yeast (Lehmann & Nielsen, 1939; Salvesen, 1940; Braendstrup, 1940). Sydenstricker (1941) pointed out that, in deficiencies of the individual vitamins of the B group, treatment with one vitamin may be rapidly followed by the development of severe signs of deficiencies of others. Klopp, Abels & Rhoads (1943) found no clinical signs of riboflavin deficiency in subjects on normal diets receiving large doses of aneurin, but found an increased urinary excretion of riboflavin. Increased excretion of riboflavin in the urine in chronic and acute aneurin deficiencies was observed by Sure (1944). Unna & Clark (1942) were

unable to show that administration of large amounts of the individual vitamins of the B group to rats deficient in one or more vitamins of this group aggravated symptoms of the specific deficiency. Sure & Ford (1942) observed a marked drop in the aneurin and riboflavin concentrations of organs and tissues of rats deprived of aneurin, while in riboflavin deficiency a drop in riboflavin content only was observed. Further, deprivation of aneurin interfered with riboflavin utilization, deprivation of riboflavin did not affect the utilization of aneurin. The efficiency of utilization of aneurin and riboflavin was also affected in deficiencies of these two vitamins (Sure & Ford, 1943). Similar studies (Russell & Nasset, 1941) indicated that pantothenic acid may play a role in the absorption of other B vitamins. The riboflavin concentration in the livers of rats deficient in one or more of the B vitamins increased during digestion and assimilation as a result of mobilization from other tissues (Supplee, Jensen, Bender & Kahlenberg, 1942). Pantothenic acid had a direct and specific function in this mobilization. Aneurin depletion was shown to diminish the process, while pyridoxine exerted no influence. Singher, Kensler, Levy, Poore, Rhoads & Unna (1944) have reported an increase in riboflavin concentration in the livers of aneurin depleted rats, and an increase in aneurin concentration in the livers of riboflavin-deficient rats.

The present investigation was undertaken in order to study interrelationships among certain vitamins of the B group in aneurin, riboflavin and biotin deficiencies, and, secondly to find out whether these interrelationships hold good with enzymes, the prosthetic group of which is one or other of the B vitamins. The enzymes selected for this study were

described by Potter & Elvehjem (1936), kept cool by ice water. The homogenized solution was passed through fine cloth and the residue homogenized again. Final volumes of the suspension were so adjusted that 1 ml contained 100 mg of the wet tissue. The suspension was kept in ice between experiments and only used during the day of its preparation. The hearts of a number of rats were pooled to obtain enough suspension for a set of experiments. In every experiment the weight of the dried tissue was determined.

General methods. Livers from the experimental and control rats were used for the estimation of vitamins. Aneurin was estimated by a thiochrome method (Bhagvat, 1943), riboflavin fluorimetrically and nicotinic acid by the cyanogen bromide method of Swaminathan (1942*a, b*).

The activities of the enzymes, aldehyde oxidase, lactic and succinic dehydrogenases, were estimated by the method of Thunberg (1920) with methylene blue as the hydrogen acceptor. The reactions were carried out at 37° in ordinary test tubes into which purified melted vaseline was poured over the reaction mixture. Owing to the absence of Thunberg tubes due to war conditions, it was necessary to devise an alternative technique to obtain anaerobic conditions. Various methods were tried, the most reproducible results being obtained with melted vaseline, which solidified in a few seconds and acted as a seal, thereby establishing anaerobic conditions. The results of this technique were comparable with those obtained when Thunberg tubes were used. The different solutions were added in the order: substrate, KCN, buffer, water, enzyme suspension and methylene blue. The tubes were shaken, and after the addition of melted vaseline were left at 37°. The time required for the decolorization of methylene blue was noted. Experiments were always set up in duplicate and with proper controls. The activity of the enzyme is expressed by the volume of O₂ (in μ l) equivalent to methylene blue reduced in the presence of substrate in 1 hr by 1 mg of dried tissue.

Preliminary trials were carried out to find out the optimum conditions for the activity of the various enzymes. The relative amounts of the substrate, enzyme and methylene blue used in these studies are given in Table 1.

Table 1 *Optimum conditions for measurement of enzyme activities*

Enzyme	Suspension (ml)	KCN M/15 (ml)	Methylene blue (0.1%) (ml)	Substrate	Water (ml)	Phosphate buffer (pH 7.4) (ml)	Final volume (ml)
Aldehyde oxidase	1	—	0.4	0.1 ml M acetaldehyde	0.5	1.0	3
Lactic dehydrogenase	1	0.4	0.2	0.1 ml M sodium lactate	0.3	1.0	3
Succinic dehydrogenase	0.5	—	0.2	0.2 ml M sodium succinate	2.1	1.0	4

the aldehyde and pyruvic oxidases and the lactic and succinic dehydrogenases. The effect of deficiency of certain members of the B group of vitamins on the activities of these enzymes has been investigated.

EXPERIMENTAL

Estimation of enzymic activity of tissues

Preparation of tissue suspension. The organs (liver, kidney and heart) from freshly killed rats (rats were killed by a blow on the head) were dissected out and weighed in an ice-cold watch glass. They were ground in an ice-cold mortar with cold 0.1 M phosphate buffer (pH 7.4). The suspension was homogenized for 2–3 min in a homogenizer of the type

KCN was incorporated in the lactic dehydrogenase system to prevent inhibition of this enzyme by pyruvic acid, formed by oxidation (Green & Brosteaux, 1936).

The activity of the pyruvic oxidase was determined by the ability of the tissue to oxidize pyruvic acid *in vitro*. The homogenized tissue suspension (1 ml) was incubated with 50 mg of pyruvic acid at 37° for 1 hr. The pyruvic acid remaining in the reaction mixture was estimated by the method of Lu (1939). The results are expressed as mg of pyruvic acid removed by 100 mg dried tissue in 1 hr.

Animal experiments

Young rats weighing 45–65 g were caged individually over a wide mesh screening and were fed *ad lib* on the

Table 2 *Composition of the experimental diets*

Component	Aneurin deficient (%)	Riboflavin deficient (%)	Biotin deficient	
			Experimental (%)	Control (%)
Sucrose (purified)	58.0	58.0	54.0	54.0
Casein (purified)	18.0	18.0	15.0	15.0
Salt mixture* (cf. McCollum & Davis, 1913)	4.0	4.0	4.0	4.0
Autoclaved brewer's yeast	10.0	Nil	Nil	Nil
Alkali autoclaved brewer's yeast	Nil	10.0	Nil	Nil
Dried raw egg white	Nil	Nil	15.0	Nil
Dried autoclaved egg white	Nil	Nil	Nil	15.0
Gingelly oil	8.0	8.0	8.0	8.0
Dried ox liver	Nil	Nil	2.0	2.0
Shark liver oil	2.0	2.0	2.0	2.0
	100.0	100.0	100.0	100.0

Vitamin supplements/rat/day

	Aneurin deficient (μ g)		Riboflavin deficient (μ g)		Biotin deficient (μ g)	
	Control	Experimental	Control	Experimental	Control	Experimental
Aneurin	5	Nil	20	20	20	20
Riboflavin	Nil	Nil	45	Nil	45	45
Pantothenic acid	Nil	Nil	100	100	100	100
Pyridoxine	Nil	Nil	20	20	20	20

* The composition of the salt mixture used was as follows: calcium lactate (30.00 g), calcium phosphate (16.20 g), potassium phosphate (28.62 g), iron citrate (3.54 g), sodium chloride (5.19 g), magnesium chloride (7.98 g), sodium phosphate (10.41 g), potassium iodide (0.50 g).

different basal diets. The rats were distributed equally between the two sexes. The composition of the different basal diets used is shown in Table 2.

Sucrose was purified by repeated washings with 95% ethanol. Casein was purified by repeated solution in alkali and precipitation with dilute acid. The precipitated casein was washed free from acid and then with ethanol and dried in the sun. The dried casein was refluxed with 95% ethanol for 24 hr.

For experiments on aneurin deficiency, autoclaved yeast was prepared by autoclaving brewer's yeast at 15 lb pressure for 6 hr in layers not exceeding $\frac{1}{2}$ in. and subsequently drying in the sun. For riboflavin deficiency studies, dried brewer's yeast was moistened with water and brought to pH 11.0. The wet mass, spread in thin layers, was then autoclaved at 15 lb pressure for 6 hr and dried in the sun.

Egg white was prepared from hens' eggs and was dried in the sun. Autoclaved egg white was prepared by autoclaving hens' eggs at 15 lb pressure for 30 min. and subsequently drying in the sun. This treatment inactivated avidin, the antibiotic principle of egg white.

The different experimental diets were mixed and given to the animals in small trays. The experiments were repeated on three separate batches of 60 rats. The positive controls, i.e. those receiving all the vitamins, grew steadily while the negative controls grew for a time, then lost weight and developed symptoms characteristic of the particular deficiency under experiment. The time required for the onset of these symptoms varied in different experiments. Thus, the symptoms of aneurin deficiency manifested themselves in 3-4 weeks, those of riboflavin deficiency in 6-8 weeks and those of biotin deficiencies in 8-10 weeks. The animals were kept on the deficient diets till severe signs of deficiency developed. Deficient and control animals were sacrificed simultaneously and their tissues analysed for their vitamin content and enzymic content by the methods described above.

RESULTS

The effect of aneurin, riboflavin and biotin deficiencies on the aneurin, riboflavin and nicotinic acid concentrations in rat livers was studied. The relevant data are presented in Table 3.

Significant differences in the vitamin contents of the liver were observed when rats were fed the different experimental diets. Depletion of aneurin was found to increase the concentration of riboflavin in the liver above that of control animals, while in riboflavin deficiency concentration of aneurin in the liver was higher than in control animals. These observations confirm the findings of Singher *et al.* (1944). The aneurin and riboflavin concentrations in the livers of the rats maintained on diets deficient in the respective vitamins decreased rapidly during the deficiency period. Livers of aneurin and riboflavin deficient rats contained smaller amounts of nicotinic acid than the livers of control animals. Aneurin, in the liver of the rats maintained on diet deficient in biotin, showed marked decrease. A considerable difficulty was experienced in aneurin estimation in these series of experiments owing to the presence in the liver of certain fluorescent substances. The interference encountered was greater with deficient livers, while riboflavin showed an increase over that present in the livers of the control animals. Singher *et al.* (1944) did not find any significant difference in the concentrations of aneurin and riboflavin in the livers of control and biotin-deficient rats.

Table 3 *Effect of aneurin, riboflavin and biotin deficiencies on the aneurin, riboflavin and nicotinic acid content of rat livers*

Deficiency		Aneurin ($\mu\text{g}/\text{mg}$)		Riboflavin ($\mu\text{g}/\text{mg}$)		Nicotinic acid ($\mu\text{g}/\text{g}$)	
		Range	Mean	Range	Mean	Range	Mean
Aneurin	Control	2.00-7.00	3.66	13.50-28.30	21.56	48.30-100.00	75.26
	Deficient	0.40-1.60	0.83	16.30-43.00	27.70	36.30-85.70	63.94
Riboflavin	Control	1.60-8.00	4.72	15.00-35.30	22.66	29.00-150.00	75.24
	Deficient	3.60-15.50	6.88	5.00-13.10	9.76	42.80-94.00	61.30
Biotin	Control	0.80-5.50	2.14	19.80-34.80	27.77	25.00-155.00	60.78
	Deficient	0.00-2.00	0.69	25.90-49.50	37.12	37.00-120.00	59.18

Statistical analysis of the results							
Vitamin		Mean (mg/g)*		Difference of means (a) - (b)	Statistical ratio (d or t)†	5% level of significance of the statistical ratio	Remarks
		Control (a)	Deficient (b)				
Aneurin series	Aneurin	3.66 (18)	0.83 (24)	2.83	8.98 (d)	2.10	Significant
	Riboflavin	21.56 (18)	27.70 (43)	6.14	2.97 (t)	2.00	"
	Nicotinic acid	75.26 (17)	63.94 (25)	11.32	2.31 (t)	2.02	"
Riboflavin series	Aneurin	4.72 (46)	6.88 (37)	2.16	4.30 (d)	1.97	"
	Riboflavin	22.66 (34)	9.76 (35)	12.90	14.09 (d)	1.97	"
	Nicotinic acid	75.24 (39)	61.30 (33)	14.00	2.01 (d)	1.97	"
Biotin series	Aneurin	2.14 (24)	0.69 (24)	1.45	5.8 (d)	2.06	"
	Riboflavin	27.77 (24)	37.12 (24)	9.35	5.66 (t)	2.02	"
	Nicotinic acid	60.78 (22)	59.18 (20)	1.60	0.18 (t)	2.02	Not significant

* The figures in brackets represent the number of experimental animals

† $d = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(s_1^2 + s_2^2)}}$, where \bar{X}_1 and \bar{X}_2 are the two mean values, and s_1^2 and s_2^2 are the respective standard errors of the means $t = \frac{\bar{X}_1 - \bar{X}_2}{s}$, s is the error of the difference calculated on the assumption that the standard deviations of the observations within the control group and the deficient group are not significantly different from each other. The letters (d) and (t) in brackets in this column represent the formula used for calculating the statistical ratio

The activities of certain enzymes of rat tissues in aneurin, riboflavin and biotin deficiencies were next investigated. The results are illustrated in Tables 4 and 5.

Marked variations have been observed in the aldehyde oxidase content of livers and that of lactic dehydrogenase content of liver and kidneys of control rats in the three experiments. The control rats in the first experiment (aneurin deficiency) received only aneurin as a supplement, while in the other two experiments (riboflavin and biotin deficiencies respectively) in addition to aneurin, other vitamins, viz. riboflavin, pyridoxine and pantothenic acid, were given as supplements to control rats. The addition of these vitamins appears to influence the concentrations of the aldehyde

oxidase and the lactic dehydrogenase in the liver and that of lactic dehydrogenase in the kidney of rats.

In the livers from aneurin deficient rats, the activity of the aldehyde oxidase was only 60% of that found in control rats. The activity of the succinic dehydrogenase in deficient tissues, however, was found to increase by 22% in the liver, 18% in the kidney and 42% in the heart over that found in control rats. Axelrod, Potter & Elvehjem (1942) have suggested that riboflavin is the prosthetic group of succinic dehydrogenase. Since in rats the depletion of aneurin was found to increase the concentration of riboflavin in the liver (heart and kidney were not analysed for their vitamin content) the increased succinic dehydrogenase activity might be attributed to the availability of bigger amounts of

Table 5 *Effect of riboflavin and biotin deficiencies on the pyruvic oxidase activity of rat liver*

		Pyruvic acid (μg) removed by 100 mg dried liver/hr	
Deficiency		Range	Mean
Riboflavin	Control	122 90-183 10	150 28
	Deficient	75 10-137 00	103 30
Biotin	Control	129 50-195 50	154 73
	Deficient	112 00-183 00	142 24

Statistical analyses of the results

	Mean*		Difference of the means	Statistical ratio (t)†	5% level of significance of statistical ratio	Remarks
	Control	Deficient				
Riboflavin series	150 28 (20)	103 30 (20)	46 98	8 34	2 02	Significant
Biotin series	154 73 (29)	142 22 (22)	12 49	2 81	2 01	„

* The figures in brackets represent the number of experimental animals

† $t = \frac{\bar{X}_1 - \bar{X}_2}{s}$, s is the error of the difference calculated on the assumption that the standard deviations of the observations within the control group and the deficient group are not significantly different from each other

the prosthetic group. The activity of the lactic dehydrogenase in the livers of deficient rats did not vary significantly from that of livers of control rats.

In riboflavin deficiency, the activities of all the enzymes in the liver, kidney and heart were found to be markedly affected. Thus, in the livers, the activities of aldehyde oxidase, succinic and lactic dehydrogenases were only 18, 60 and 20% respectively of those found in the control animals. The activities of lactic and succinic dehydrogenases of the heart and kidney were also found to be significantly decreased. This diminution might be regarded as a result of the lowered amount of the prosthetic group (riboflavin) available. A similar fall in the activity of enzymes with riboflavin as the prosthetic group, e.g. xanthine oxidase (Ball, 1938, 1939, Corran, Dewan, Gordon & Green, 1939), succinoxidase and D amino acid oxidase (Axelrod & Elvehjem, 1941, Axelrod, Potter & Elvehjem, 1942, Axelrod, Swingle & Elvehjem, 1942, Axelrod, Sober & Elvehjem, 1939), has been reported. The effect of riboflavin deficiency on the concentration of coenzymes and other factors necessary for the complete oxidation of certain metabolites, viz. aldehyde, lactate, etc. was not investigated.

In biotin deficiency the oxidation of aldehyde by the liver was decreased below that of control rats, while that of lactate was increased. The succinic and lactic dehydrogenases of heart and kidney were found to be unaffected in biotin deficiency. This confirms with the findings of Axelrod *et al.* (1939). The pyruvic oxidase activity of rat liver in ribo-

flavin and biotin deficiency was decreased. This might be due to partial depletion of the two vitamins in the tissues, since it has been reported that riboflavin and biotin are essential for the complete oxidation of pyruvic acid (Lipmann, 1939, Pilgrim & Elvehjem 1944, Pilgrim, Axelrod & Elvehjem, 1942).

In the oxidation of acetaldehyde, all the three vitamins appear to be involved, since withdrawal of any one of these decreases the activity of the aldehyde oxidase, though its prosthetic group has been shown to be riboflavin (Subrahmanyam, Gordon & Green, 1939).

The results in general show that the activity of the different enzymes studied depends on the relative concentrations of the different B vitamins, thereby indicating a close interrelationship between them.

SUMMARY

1. Interrelationships between the vitamins of the B group in rats in aneurin, riboflavin and biotin deficiencies have been studied.

2. The riboflavin concentration in the liver was found to be increased in aneurin deficient rats, while riboflavin deficient rats had an increased concentration of aneurin in the liver. In biotin deficiency, the concentration of riboflavin in the liver was increased, while that of aneurin decreased. Livers from aneurin deficient and riboflavin-deficient rats contained a smaller amount of nicotinic acid than that present in livers of control rats.

3. The activities of aldehyde oxidase, lactic and succinic dehydrogenases depends on the relative

concentration of aneurin, riboflavin, nicotinic acid and biotin in the tissues. Increase in the concentration of riboflavin in the liver increases the activity of the lactic and succinic enzymes (with the exception of lactic dehydrogenase of aneurin deficient livers), while a decrease in the riboflavin content decreases their activity. Similar results have been obtained for lactic and succinic dehydrogenases of heart and kidney in riboflavin series and for succinic dehydrogenase in aneurin series, while the enzyme make up of these tissues has been shown to be unaffected in biotin series.

4 The oxidations of acetaldehyde and pyruvic acid appear to involve all the vitamins, aneurin, riboflavin and biotin, since withdrawal of any one of these appears to decrease the activities of their respective enzymes.

5 The results are interpreted as evidence of an interrelationship between certain vitamins of the B group.

The authors are grateful to Mr S J Poti of the Department of Statistics, All India Institute of Hygiene and Public Health, Calcutta, for the statistical analysis of the results presented in the paper.

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The Fractionation of Weak Electrolyte Mixtures by Ion-Exchange Resins

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(Received 31 December 1948)

Ion-exchange resins are becoming of increasing importance in the separation of mixtures of weak electrolytes, and the object of the present paper is to enumerate some general considerations which are of importance in the planning and interpretation of separation experiments.

Choice of resin. Numerous experiments in these laboratories and elsewhere (see, for instance, Partridge* & Brimley, 1949) have established the fact that processes which involve the ionization of weakly acidic or basic groups in the resin structure are, com-

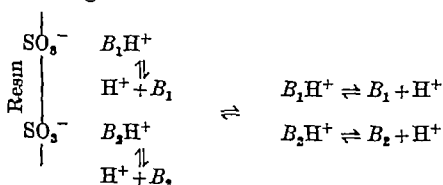
paratively, very slow, they also involve large swelling changes. The reverse processes are equally slow, probably because it is the surface of the granules that will be first attacked by a fresh reagent, and a reduction of charge and a desorption of water in the surface layers will hinder the diffusion of material to and from the interior of the gel. Procedures involving an alteration in the charge of the resin are therefore not well suited to chromatographic separations. For the types of resin in common use this means that cationic exchangers containing phenolic as well as sulphonic acid groupings should not be used at pH values greater than 8, and that the weakly acidic or weakly basic resins should if possible be employed

* The author is much indebted to Dr Partridge for an early view and discussion of his recent papers.

only for exchange processes in which the pH can be maintained at a suitable constant value

Whatever the type of resin, separations will be the sharper the more nearly the processes at the resin surface approach to a state of equilibrium. Separations will, therefore, be improved by reducing the rate of flow and the grain size, and by working at elevated temperatures (within the range of stability of the resin) in jacketed columns

Separations through salt formation The separation of two weak bases B_1 and B_2 may be taken as a typical example. If these are applied, as such, to a column of sulphonic acid type resin they will be quantitatively adsorbed $R\text{SO}_3^- \text{H}^+ + B = R\text{SO}_3^- \text{BH}^+$, and subsequent development with a stronger base B_3 will give an effluent in which the bases should appear in the order of increasing basic dissociation constant. Conditions during the separation are illustrated in the following scheme



The two cations initially adsorbed on the resin will be in reversible equilibrium with their dissociation products in the surface layer, and this system will be in equilibrium with the solution remote from the surface. On admitting the developing solution the new base will react with the hydrogen ions, and the cations previously held by the resin will be displaced, B_1 and B_2 will move down the column as uncharged molecules only to be adsorbed at fresh sulphonic acid groups and the whole process repeated. A separation will clearly require that the bases, in their movement down the column, are retarded by the resin to different extents. This retardation will depend on two factors, the strength of the attraction of the resin for the cation BH^+ and the dissociation constant of the base.

The first of these factors will be of primary importance if the cations differ in charge, bivalent ions, for instance, are much more strongly adsorbed than univalent ions of similar structure. If, on the other hand, the ions are of the same valency, and, assuming that the adsorption affinity is mainly electrostatic, this factor may be expected to be unimportant, and the separation will depend almost entirely upon a difference in dissociation constants. Quite a small difference is effective, complete separations have been achieved of constituents differing in pK by less than 0.2 unit (see the last section).

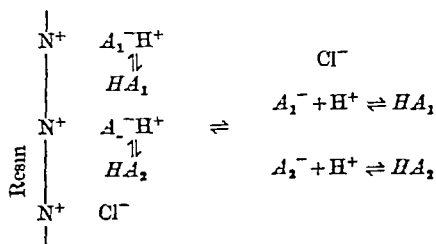
If the bases to be separated are of the same valency and of identical dissociation constant, a successful fractionation may still occur if differences are appreciable in the affinity of the resin for the various

cations. The adsorption affinity may be regarded as made up of two parts, an electrostatic term and a 'van der Waals' term. The first of these makes an important contribution to the total adsorption energy, but one which is unlikely to show sufficiently large variations to be made the basis of separations. Variations in the 'van der Waals' term, on the other hand, may well be important, and information on this point can be gained from adsorption measurements using the uncharged electrolyte. The data so far available (Bhatnagar, Kapur & Bhatnagar, 1939, Thomas, unpublished) show that molecular adsorption on resins is often very large, that it is particularly marked with aromatic compounds, and that in a homologous series it increases as expected in molecular weight, the same rules should apply to the corresponding ions, which differ in chemical structure by only one proton. Separations believed to be based on this effect are quoted in the last section.

In the preceding paragraphs differences in pK and differences in adsorption affinity have been discussed separately. They will of course be superimposed, and in some cases the two factors may be of comparable importance. It should be added, also, that a very high adsorption affinity, such as is common with aromatic compounds, will in general have an adverse effect on separations. This is because the uncharged electrolyte travelling down the column will be adsorbed to a significant extent, and, as molecular adsorption and desorption are very slow processes compared with ion exchange, this will result in the highly adsorbed constituent being spread out on the column in the rear of its advancing band. It should be possible to minimize this effect by operating at elevated temperatures.

Separations depending upon ion exchange With the advent of strong base resins the technique of displacement development discussed in the second section will become applicable to the separation of weak acids. There are separations, however, for which weak acid or weak base resins will be employed, and, as was pointed out in the first section, it will be best to restrict the use of these to exchange processes at a constant pH.

Let HA_1 , HA_2 be two weak acids to be separated on a weak base resin. The resin will first be conditioned with, say, hydrochloric acid of the pre-determined pH. On adding the acids conditions will be somewhat as shown below



In a case where the adsorption affinities for A_1^- and A_2^- do not differ greatly, the separation of the acids will depend on a difference in the dissociation constants K_{A_1} and K_{A_2} . If activity coefficients, which will be virtually equal for the two anions, are neglected, the proportion of the first acid which is present in the form of anion can be written as

$$\frac{K_{A_1}}{K_{A_1} + [H^+]}, \text{ and similarly for the second. It can then be}$$

readily shown that these ionized fractions show their maximum difference when $pH^* = \frac{1}{2}(pK_{A_1} + pK_{A_2})$, and this condition gives the optimum pH for the separation. It must be noticed, however, that the argument applies to conditions at the surface of the resin, and here the ionization equilibrium is greatly modified by the surface charge. The effective pH to be used in the above equation will be given by

$$pH^* = pH + \frac{V}{0.059}, \text{ where the first term on the right}$$

hand side is the pH of the solution remote from the resin surface, and V is the potential difference in volts through which a hydrogen ion must be moved in bringing it from the remote solution up to the surface of the adsorbed anion. In general, the value of V will be unknown, and even for the same resin it will vary with the pH and the ionic strength of the solutions employed. However, Hartley & Roe (1940), who have derived the above equation and applied it to a similar problem in measuring indicator shifts at the surface of a colloidal micelle, found values of V varying over the range 50–100 mV, and work in progress here on the electrokinetic potential at resin surfaces leads to similar values. It can be anticipated, therefore, that in working with a positively charged resin the best separation of two anions will be achieved when the pH of the solutions applied to the resin is 1–2 units lower than the value of $\frac{1}{2}(pK_{A_1} + pK_{A_2})$. In separating weak bases at a negatively charged resin the corresponding condition is that the pH of the solutions used should be 1–2 units higher than the value of $pK_w - \frac{1}{2}(pK_{B_1} + pK_{B_2})$, where K_{B_1} and K_{B_2} are the basic dissociation constants of the substances to be separated.

Interesting confirmation of these conclusions is provided by the work of Consden, Gordon & Martin (1948), who succeeded in separating glutamic from aspartic acid on Amberlite IR 4. These authors point out that the best separation would be expected at pH 4, but find that actually the solutions must be applied to the resin at pH 2.5, a value at which the acids carry a small net positive charge.

Separation of amino-acids Partridge (1949) has had striking success in the separation of amino acids on a resin column. The results appear to be in excellent agreement with the considerations advanced in the second section of this paper, and to throw some light on the relative importance of the factors discussed there. Partridge applied the amino acid

mixture as hydrochlorides to a column of sulphonic acid resin. The hydrogen ion concentration at the resin surface was therefore high, and the monoamino acids were present largely in the form of univalent cations, whilst the diamino acids, including cystine, were present partly as bivalent cations, and consequently were much more firmly held at the resin surface. On displacement development with ammonia, fractions were collected in the order shown below.

(The figures given after the names of the acids are the negative logarithms of the acid dissociation constants for the reaction $A^+ \rightleftharpoons A^\pm + H^+$, taken from Cohn & Edsall (1943), that is to say, they are the pK_1 values for the monoamino acids and the pK_2 values for the diamino acids, these dissociation constants are primarily responsible for controlling the movement of acid molecules down the column. The pK of threonine, which is not listed, may be presumed to lie close to that of serine.)

Fraction

- I Aspartic acid, 1.88
- II Glutamic acid, 2.19, serine, 2.21, threonine
- III Glycine, 2.34, alanine, 2.34
- IV Valine, 2.32, proline, 1.99
- V Leucine, 2.36, isoleucine, 2.36, methionine, 2.28, cystine $pK_1 = 1.0$, $pK_2 = 2.0$
- VI Histidine, $pK_2 = 6.00$
- VII Lysine, $pK_2 = 8.95$
- VIII Arginine, $pK_2 = 9.04$ (this is not displaced by dilute ammonia)

The monoamino acids come through first, followed by the diamino acids, and with two exceptions the order throughout is that to be anticipated from the pK values. Proline falls out of place, being more firmly held on the resin than would be expected from its pK value, but in view of its cyclic structure it is reasonable to attribute this anomaly to a specially large contribution from the 'van der Waals' adsorption factor. The other exception is cystine, where the low pK_2 value (which by itself would lead to the appearance of cystine in the earliest fractions) is offset by the existence of a considerable portion of the acid at low pH values in the form of bivalent cations.

Some numerical values may serve to illustrate the argument. Partridge (1949) found that the early fractions emerged from the column at pH of about 3, and the effective pH at the resin surface during a large part of the separation may be taken as being round about 1–2. In a mixture of amino acids at pH 1.5, only 70% of the aspartic acid will be in the form of cations, the corresponding figures for serine and leucine are 83 and 88% respectively. Cystine will be 20% un-ionized and only 80% in the form of cations, but a quarter of these latter will be carrying two positive charges, and will require to lose two protons before being displaced from the resin.

surface, the net rate of movement down the column may therefore be expected to be comparable with that of leucine. Finally, histidine and the other diamino acids will be completely ionized and firmly held by the resin at this pH.

Fractions III, IV and V consist of acids whose pK values are so close that a separation depending on this factor would not be expected, and according to the argument given in the second section we should look for an explanation in terms of adsorption affinity. The molecular weights of the acids concerned are

Fraction

III Glycine, 75 alanine, 89

IV Valine, 117

V Leucine, isoleucine, 131, methionine, 149

The results do, therefore, seem to provide real evidence that, in the absence of significant pK differences, separations based on quite small differences in chain length and adsorption affinity can be carried out on ion-exchange columns.

Partridge (1949) found that tyrosine and phenylalanine showed anomalous behaviour, and had to be removed in a preliminary operation. This is in keeping with the strong adsorption of all aromatic compounds, a factor which seems at present to set the most important limitation to the use of ion-exchange resins.

SUMMARY

The theory of the separation of weak electrolytes by ion exchange resins is discussed and illustrated by reference to recent work with the amino acids.

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Direct Transformation of Fumarate to Oxaloacetate, without Intermediate Formation of Malate, by *Clostridium saccharobutyricum*, Strain GR 4

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(Received 3 January 1949)

Annau, Banga, Goszy, Huszák, Laki, Straub & Szent Gyorgyi (1935), on the basis of differences between fumarate and malate oxidation, thought that fumarate might be oxidized without intermediate formation of malate. Later, Laki (1936) abandoned this view. However, Kalekar (1939) showed that kidney extracts can dehydrogenate an addition product of fumarate and phosphate with formation of phosphoenoloxaloacetate. Lipmann (1941) assumed that the earlier view of Annau *et al.* (1935) was in part correct, and that the differences between the oxidation of the two substrates could probably be explained by the co-existence of two different paths of fumarate breakdown, the first occurring via malate, the second leading directly to oxaloacetate through a phosphorylated derivative of fumarate. This hypothesis is supported by the observation of Lipmann (1942) that, in a similar manner, the transformation of crotonate to aceto-

acetate occurred in rabbit kidney without intermediate formation of β hydroxybutyrate.

Clifton (1942), studying the fermentation of C_4 dicarboxylic acids by washed suspensions of *Clostridium tetani*, found that fumarate is partly fermented to butyrate and acetate and partly to lactate, malate and ethanol. Malate and succinate were not fermented. Pickett (1943), with the same organism, showed that if incubation is continued for 4 days, malate is slowly fermented with production of acetate and butyrate, ethanol, carbon dioxide and succinate. The latter was not fermented. Cohen, Nisman & Cohen Bazire (1948) have shown that citrate and α ketoglutarate are slowly fermented to butyrate and acetate by strain GR 4 of *Cl. saccharobutyricum*. It is very unlikely that these two compounds are broken down via the C_4 -dicarboxylic acids system, as it will be shown that succinate is not fermented by this organism.

Further work with this organism, reported in the present paper, shows that it is improbable that the breakdown of fumarate occurs via malate, and evidence is presented in support of an alternative scheme

EXPERIMENTAL

Organism

The organism, named according to Prévot (1948), is a variety of *Cl. butyricum* Prazmowski. Prévot, who has given us this strain, names it *Cl. butyricum*, var *saccharobutyricum* Schattenfroh & Grassberger. The strain GR4 of this organism, which has been isolated from river mud, is a small thin rod, with subterminal spores, motile, Gram positive, its dimensions vary from $3-12 \times 0.8-1 \mu$. Deep agar colonies appear as dense cottony masses. Gelatin is liquefied after 7 days. Milk is coagulated in 5 days, the clot is further fragmented, but not digested. Indole is formed. All the sugars tested were fermented except galactose, arabinose, dulcitol and inulin. Butyrate, acetate and lactate were formed when the organism was grown on VF broth (peptic digest of muscle and liver) containing 0.2% glucose. Acetone and acetylmethylcarbinol were not formed under these conditions. Cohen & Cohen Bazire (1948a, b) have separated the butyric bacteria into two biochemical types according to their ability, in washed suspension, to ferment pyruvate with production of (a) acetate and butyrate ('normal' strains), or (b) acetate only ('deficient' strains). The GR4 strain is 'normal', lactate is also formed, but in the present work attention is confined to the volatile acids.

Preparation of cell suspensions

The organism was grown on VF broth, of which 1 l. was inoculated with 5 ml of an 18 hr subculture and incubated for 20 hr at 37°. The cells from 1 l of culture were centrifuged out, washed twice with 15 ml of previously de-aerated saline and finally resuspended in approx 25 ml of saline. All operations were carried out with sterile precautions. A quantity of suspension (3 ml.), corresponding to approx 8 mg bacterial N, was taken for each experimental tube. The yield of cells varied only slightly from one culture to another.

Procedure and methods

Chemicals These were obtained from the sources indicated. Oxaloacetic acid (prepared as described by Fenton & Jones, 1900), succinic acid (Merck), fumaric acid, malic acid, hydroxylamine hydrochloride and sodium arsenite (Pro-labo), sodium pyruvate (Hofmann la Roche), congo red (R.A.L.). Solutions of the acids and of hydroxylamine hydrochloride were neutralized before use.

Fermentations All operations up to the completion of the fermentation were carried out with sterile precautions in view of the long period of incubation (24 hr). Substrates and inhibitors were sterilized by filtration (L_4 Chamberland candles), water and buffer by autoclaving. Substrates, bacterial suspension, phosphate buffer (pH 7, final conc 0.033M) were measured into test tubes and brought to a total volume of 20 ml with water. The tubes were evacuated, sealed and incubated for 24 hr at 37°. The total contents of each tube were then acidified with 5 ml of 40% (w/v) tartaric acid and steam distilled, 130 ml. of distillate were

collected. A portion (20 ml.) of the distillate was used for the determination of total volatile acids by titration with 0.1N-NaOH. The remaining 110 ml. were used for the determination of the nature and proportions of these acids by the Duclaux (1895) method. Our Duclaux calibration curves, performed with redistilled acids, differed little from the original Duclaux curves.

The method of Raynaud & Gros (1947) was used for the determination of NH_3 in the experiments in which NH_4OH was used. In the same experiments, one dimensional paper chromatography (Whatman no 1 paper, solvent, 95% phenol) was used for the detection of aspartic acid (Consden, Gordon & Martin, 1944).

RESULTS

Comparative rate of fermentation of succinate, fumarate, malate, oxaloacetate and pyruvate

The results recorded in Table 1 show that volatile acids are produced from these substrates at a rate which decreases in the order pyruvate > oxaloacetate > fumarate > malate. Succinate is not

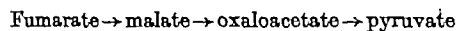
Table 1. Fermentation of succinate, fumarate, malate, oxaloacetate and pyruvate

(Each tube, sealed *in vacuo*, contained 4000 μmol of substrate and 8 mg bacterial N. Total vol 20 ml, pH 7, 37°. Incubation 24 hr.)

Exp no	Substrate	Total volatile acid (μmol)	Acetic acid (μmol)	Butyric acid (μmol)
1	Fumarate	780	478	302
	Malate	390	312	78
	Pyruvate	936	468	468
2	Succinate	0	0	0
	Fumarate	562	315	247
	Malate	361	281	70
3	Pyruvate	940	470	470
	Succinate	0	0	0
	Fumarate	655	406	249
4	Oxaloacetate	842	505	237
	Pyruvate	611	409	202
5	Succinate	0	0	0
	Fumarate	390	260	130
	Malate	250	200	50
6	Malate (4 days)	671	505	134
	Oxaloacetate	1014	639	375
7	Fumarate	562	264	208
	Oxaloacetate	795	452	328
	Pyruvate	2184	1245	939
8	Fumarate	702	435	267
	Malate	499	375	124
	Oxaloacetate	780	460	320
9	Pyruvate	1638	868	770

attacked, the organism had no succinic dehydrogenase activity. With malate the relative proportion of butyric and acetic acids differs markedly from that obtained from the other substrates, much less butyric acid is formed from malate. These results

make it very improbable that the classical reaction sequence



occurs in these fermentations. They suggest rather a direct conversion of fumarate to oxaloacetate, the latter then giving rise to pyruvate.

Non inhibition of fumarate fermentation by congo red

Quastel (1931, 1936-7) found, both with whole cells of *Escherichia coli* and isolated enzyme preparations, that this dye (at a concentration of $1.2 \times 10^{-5} M$) inhibits fumarase. Table 2 shows that the fermentation of fumarate is not affected by congo red. This is consistent with the view that the conversion of fumarate to malate is not a stage in the fermentation of the former by *Cl. saccharobutyricum* GR4. The dye is also without effect in the fermentation of malate, oxaloacetate, pyruvate and citrate.

Table 2 *Effect of congo red on fermentation of fumarate*

(Conditions see Table 1. Substrate fumarate, 4000 μmol)

Congo red Concentration (M)	Volatile acidity (μmol)	Nature of acids
(a) 0	780	$1/2 < \frac{\text{Butyric}}{\text{Acetic}} < 1/1$
(b) 1.2×10^{-5}	780	
(c) 2.4×10^{-5}	749	
(d) 4.8×10^{-5}	733	
(e) 2.4×10^{-4}	749	

Effect of hydroxylamine on the fermentation of C_4 -dicarboxylic acids

A further difference in the behaviour of malate, compared with fumarate and oxaloacetate, was observed in experiments in which an attempt was made to inhibit the fermentation of these substances by adding equimolar quantities of hydroxylamine. It was thought that the chain of reactions would be blocked at the oxaloacetate stage by the fixation of this keto acid as an oxime. It was found, however, that, with this organism, oxaloacetate and fumarate but not malate, could act as hydrogen donor for the reduction of hydroxylamine to ammonia. After 24 hr incubation the tubes containing hydroxylamine plus fumarate or oxaloacetate had become deep yellow, on opening there was a strong smell of ammonia. Furthermore, the steam distillate from

such tubes had an odour of acetaldehyde. This was identified by preparation of the dimedone derivative (m.p. 139°). In the case of hydroxylamine plus malate there was no formation of yellow colour, ammonia or acetaldehyde.

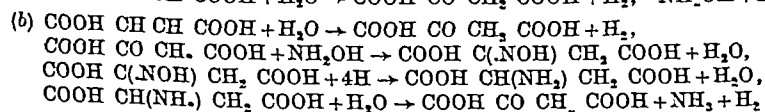
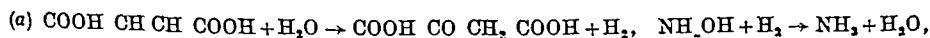
Experiments were carried out in which both total volatile acid and ammonia were estimated after the action of the organism on all three dicarboxylic acids in the presence and absence of hydroxylamine (Table 3). It will be seen that ammonia is not formed from hydroxylamine in the presence of malate, but only with fumarate or oxaloacetate. In all three cases total volatile acid is diminished by 30-100% in the presence of hydroxylamine. The rate of reduction of hydroxylamine to ammonia is apparently independent of the proportion of the dicarboxylic acid converted to volatile acids.

Table 3 *Effect of hydroxylamine on fermentation of C_4 dicarboxylic acids*

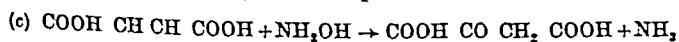
(Conditions see Table 1. Neutralized NH_2OH HCl 4000 μmol , C_4 acid 4000 μmol)

Exp no	Substrate	Volatile acidity (μmol)	NH_3 (μmol)
2	Fumarate	562	0
	Hydroxylamine	0	0
	Fumarate + hydroxylamine	280	1850
3	Fumarate	655	0
	Hydroxylamine	0	0
	Fumarate + hydroxylamine	390	1880
4	Fumarate	390	0
	Hydroxylamine	0	0
	Fumarate + hydroxylamine	120	1800
	Oxaloacetate	1014	0
5	Oxaloacetate + hydroxylamine	312	960
	Fumarate	562	0
	Fumarate + hydroxylamine	0	2000
	Oxaloacetate	795	0
7	Oxaloacetate + hydroxylamine	78	1040
	Fumarate	—	0
	Fumarate + hydroxylamine	—	1600
	Malate	—	0
8	Malate + hydroxylamine	—	0
	Fumarate	350	0
	Fumarate + hydroxylamine	—	1000
	Malate	—	0
	Malate + hydroxylamine	—	28

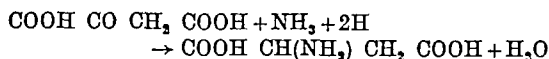
In the case of fumarate the production of ammonia can be explained in two different ways



These two series of equations represent the same overall reaction



Scheme (b) is supported by the fact that *Cl. saccharobutyricum* GR4 rapidly deaminates aspartic acid (Cohen, Nisman & Cohen Bazire, 1948). Furthermore, aspartic acid, and no other amino acid, can be detected by paper chromatography of the contents of tubes containing hydroxylamine and fumarate or oxaloacetate (Table 4). However, it is also possible that aspartic acid arises from a secondary reductive amination of oxaloacetate



It is, therefore, not yet possible to decide which of the suggested mechanisms is correct. Furthermore, Nisman (1948) has shown that washed suspensions of our strain can carry out the direct amination of fumarate in presence of ammonia. Such a reaction may also complicate the overall phenomenon. This problem is being further investigated.

It will also be seen from Table 4 that no aspartate was detected in the tubes containing hydroxylamine plus malate. Since it has already been shown that malate cannot act as hydrogen donor for the reduction of hydroxylamine, the totally different behaviour of malate again indicates that it cannot be an intermediate in the fermentation of fumarate.

Table 4 *One dimensional filter paper chromatography*

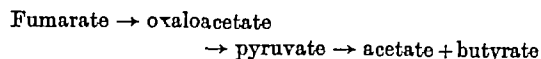
(Solvent 95% phenol Paper Whatman no 1 N_m hydram 1% butanol solution)

Substrate	R _F
(a) Fumarate NH ₂ OH	0.10
None (suspension)	—
Aspartate (blank) (10 ⁻³ M)	0.10
(b) Fumarate NH ₂ OH	0.11
Aspartate (blank)	0.11
Suspension	—
(c) Fumarate NH ₂ OH	0.11
Oxaloacetate NH ₂ OH	0.11
Aspartate (blank)	0.12
Suspension	—
Fumarate	—
Oxaloacetate	—
(d) Fumarate NH ₂ OH	0.12
Malate NH ₂ OH	—
Aspartate (blank)	0.12
Suspension	—
Fumarate	—
Malate	—
(e) Fumarate NH ₂ OH	0.12
Malate NH ₂ OH	—
Fumarate	—
Malate	—

Effect of arsenite on the fermentation of C₄ dicarboxylic acids

Cohen-Bazire, Cohen, Nisman & Raynaud (1948) have shown that the fermentation of pyruvate in the presence of 10⁻³M sodium arsenite leads to the pro-

duction of acetate only, production of butyrate was completely inhibited and total volatile acid was decreased by approx. 30%. The effect of arsenite on the fermentation of fumarate, malate and oxaloacetate was therefore tested (Table 5). Inhibition of the same type as with pyruvate was obtained. Taken in conjunction with the experiments of Table 1 these results are consistent with the following reaction sequence



The results with arsenite do not exclude the possibility that pyruvate is also an intermediate in the fermentation of malate. If this is the case, then, in view of the other results already given, malate must be converted to pyruvate by a mechanism not involving oxaloacetate.

Table 5 *Effect of arsenite on fermentation of C₄ dicarboxylic acids*

(Conditions see Table 1. Dicarboxylic acids 4000 μmol. Sodium arsenite, final concentration 10⁻³M)

Substrate	Volatile acidity (μmol.)	Acetic acid (μmol.)	Butyric acid (μmol.)
Pyruvate	1820	910	910
Pyruvate + arsenite	1560	1560	0
Oxaloacetate	663	330	303
Oxaloacetate + arsenite	598	544	58
Fumarate	858	572	286
Fumarate + arsenite	510	464	46
Malate	473	379	94
Malate + arsenite	390	390	0

DISCUSSION

The present results give experimental support to the hypothesis (Lipmann, 1941) that there may be direct transformation of fumarate to oxaloacetate without intermediary formation of malate. Clifton (1942), working with *Cl. tetani*, found that malate was not fermented, but Pickett (1943) in experiments with the same strain in which incubation was prolonged to 4 days showed that it was attacked very slowly. With *Cl. saccharobutyricum* GR4, malate is attacked at a somewhat higher rate than with *Cl. tetani*, although less rapidly than fumarate and oxaloacetate. This observation, and the different butyric acid/acetate ratio found for malate compared with fumarate excludes malate as an intermediate in fumarate fermentation. Evidence supporting this conclusion was obtained in the experiments with congo red and hydroxylamine.

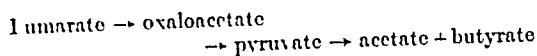
It has also been found, incidentally, that the test organism reduces hydroxylamine to ammonia in the presence of fumarate or oxaloacetate, oximin succinic acid and aspartic acid are possibly intermediates in this reaction. Although the exact

mechanism is not yet certain, the phenomenon is interesting since many butyric acid bacteria have been described as nitrogen fixers and hydroxylamine or ammonia have been proposed for other organisms as primary products of nitrogen fixation. The present experiments indicate that the hydroxylamine theory of Virtanen & Laine (1939, see also Virtanen, 1947), and the ammonia theory of Burris & Wilson (1945, 1946 see also Wilson & Burris, 1947), are not necessarily incompatible since both substances give rise to aspartic acid with either fumarate or oxaloacetate. Woods (1938) has shown that hydroxylamine is reduced by molecular hydrogen to ammonia in the presence of suspensions of *Clostridium*. It is now shown that such reduction can also occur with other hydrogen donors, this point will be further studied. Another type of reaction between hydroxylamine and fumarate leading to hydroxyaspartic acid, which is not further utilized, has been reported by Jacobsohn & Soares (1936-7) for suspensions of *Escherichia coli*.

SUMMARY

1 A study has been made of the comparative rates of fermentation of fumarate, malate, oxaloacetate and pyruvate by cell suspensions of *Clostridium saccharobutylicum* GR 4. The relative proportions of butyric and acetic acids formed were also measured.

2 The results indicate the following sequence of reactions



Malate is not an intermediate in the transformation of fumarate to oxaloacetate. This is supported by experiments with various inhibitors.

3 The organism reduces hydroxylamine to ammonia in the presence of fumarate or oxaloacetate.

We wish to express our gratitude to Dr D. D. Woods for his generous help in the preparation of the typescript.

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The Fate of Certain Organic Acids and Amides in the Rabbit

8 TOLUIC ACIDS AND AMIDES

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(Received 12 January 1949)

The metabolism of the toluic acids and their amides was investigated primarily in order to determine the effect of a nuclear substituted methyl group upon the hydrolysis of the carbamyl group *in vivo*. It was also of interest to compare the excretion products of the acids with those of the xylenes (Bray, Humphris & Thorpe, unpublished results) from which they are

derived *in vivo*. Further, since the toluamides provide examples of compounds with two different potential 'centres for conjugation' (cf. Bray, Ryman & Thorpe, 1948), the metabolic fates of these compounds could be compared with those of the xylenes, in which the two centres are identical in character.

Kraut (1856) and Gleditsch & Moeller (1889) isolated the toluric (methylhippuric) acids, $\text{Me C}_6\text{H}_4 \text{ CONH CH}_2 \text{ COOH}$, as metabolites of the toluric acids in man. Quick (1932) found that all three toluric acids gave rise to increased excretion of glucuronic acid and glycine by the dog. *o*-Toluric acid was conjugated only slightly with glycine, but to a considerable extent with glucuronic acid, a small amount was excreted unconjugated. *p*-Toluric acid was conjugated with glycine to a much greater extent than was *m*-toluric acid, but both these isomers were conjugated with glucuronic acid to a smaller extent than was *o*-toluric acid. Bernhard (1937), on the other hand, found that the dog excreted all three toluric acids unconjugated.

In this investigation the effect of the acids and amides on the excretion of ether soluble acid, reducing material and ethereal sulphate by rabbits was studied and the nature of the main metabolites determined.

MATERIALS AND METHODS

Materials. The acids used were obtained either from British Drug Houses Ltd. or from Hopkin and Williams Ltd. The amides were prepared from the corresponding nitriles (from the same sources) by the method of Noller (1933). Phthalide was prepared from phthalimide (British Drug Houses Ltd.) by the method of Reissert (1913).

Diet and dosage. The rabbits used were does of 2–3 kg and received the diet previously described (Bray, Ryman & Thorpe, 1947) throughout the investigation. The compounds were administered by stomach tube, the acids as solutions of their sodium salts and the amides as suspensions in water. The only compound which possessed marked toxic properties at dose levels up to 0.3 g/kg was *o*-toluamide. At a dose level of 0.25 g/kg this compound caused a deep narcosis lasting several hours, although the animals recovered completely. Doses of *o*-toluic acid corresponding to 0.75 g/kg were in some cases fatal and *m*-toluamide occasionally caused slight narcosis. The other compounds were without toxic effect at the dose levels used.

Quantitative methods. The methods used for the estimation of ether soluble acid, reducing material and ethereal sulphate have already been described (Bray, Thorpe & Wood, 1949). Toluene fractionation of the ether soluble acid was not carried out, since the differences in the solubilities of the toluric and toluric acids were not sufficiently well defined. The presence of labile ether soluble glucuronides which might decompose during toluene extraction would also complicate the interpretation of the results obtained.

RESULTS

Quantitative studies

Normal excretion of metabolites. The average daily output of ether soluble acid, reducing material and ethereal sulphate for individual rabbits ranged as follows: 629–852 mg (calc. as hippuric acid), 130–244 mg (as glucuronic acid) and 33–45 mg SO_2 . In sixty-one experiments the average percentages, by which the weekly average figures used as base

line values for the calculation of results differed from the individual overall averages, were, for ether soluble acid, $\pm 7\%$, and, for reducing values, $\pm 8\%$. The corresponding difference for ethereal sulphate in sixteen experiments was $\pm 8\%$ ($\pm 3 \text{ mg SO}_2$).

Metabolism of *o*-toluic acid and amide. The results obtained are given in Table 1. It can be seen that the percentages of the doses of *o*-toluic acid excreted as ether soluble acid and as ester glucuronide are approximately the same. The experiments recorded in Table 2 show that the reducing power of the ether soluble acid was, within experimental error, identical with that of the urine as determined directly. Thus ether soluble ester glucuronide appears to be the principal metabolite of *o*-toluic acid in the rabbit.

Table 1 *Metabolism of o-toluric acid and amide in the rabbit*

Rabbit no	Percentage of dose excreted as	
	Ether soluble acid	Ester glucuronide
<i>o</i> -Toluric acid (dose level 0.3 g/kg)		
134	77	70
134	67	83
216	72	64
216	71	61
216	84	61
216	78	58
221	70	71
228	84	86
228	83	86
228	78	86
228	—	80
228	—	66
229	79	81
Average	77	73
<i>o</i> -Toluamide (dose level 0.25 g/kg)		
134	46	13
134	18	—
134	10	—
168	22	9
168	17	11
168	0	—
168	29	15
190	23	—
216	37	12
216	50	—
217	0	77
227	0	—
227	42	13
227	38	15
227	31	31
228	45	16
228	0	—
229	0	6

The analytical results obtained from *o*-toluamide urine were very variable. Further, the 'extra' reducing material could not, as in the case of *o*-toluic acid urines, always be completely extracted with ether, and presumably, therefore, did not consist only of the ester glucuronide metabolite of *o*-toluic acid. In view of the toxicity of *o*-toluamide it seems possible that the extra reducing material represents a response similar to that observed with *o*- and *m*-acetotoluides (Bray & Thorpe, 1948). A similar effect has also been

Table 2 *Ether soluble nature of reducing material excreted by rabbits after administration of o toluic acid*

Rabbit no	Dose* (g)	24 hr period	Reducing value (expressed as mg glucuronic acid)			
			Urino	Urine after ether extraction (a)	Ether soluble material (b)	Total (a) + (b)
228	1	1st	253	205	12	217
		2nd	1408	298	1038	1336
		3rd	285	239	16	255
228	1	1st	199	192	15	207
		2nd	1276	281	950	1231
		3rd	251	270	24	294
228	1	1st	250	267	14	281
		2nd	1105	266	907	1173
		3rd	212	211	34	245

* Dose administered at beginning of second 24 hr period

observed in some cases after dosage with *o* xylene, when the urine contains extra reducing material which is insoluble in ether, in addition to the usual amount of ether soluble reducing material (presumably *o* toluylglucuronic acid) which is excreted alone in the majority of the experiments (Bray *et al* unpublished results) *o* Toluamide is the only compound of the six studied which had any effect on the ethereal sulphate excretion, but this effect was very small and almost within experimental error

Metabolism of m toluic acid and amide Table 3 summarizes the results obtained. The percentages of the doses of acid and amide excreted as ether soluble acid are comparable, but the

extent of glucuronide conjugation is much less in the case of the amide. The quantitative results considered without reference to the nature of the metabolites of the amide suggest that the carbamyl group is completely hydrolysed, whereas, as will be described later, the acid arises only in part from the hydrolysis of the amide group, the remainder being the result of oxidation of the methyl group. The ester glucuronide is completely extracted by ether and is, therefore, included in the ether soluble acid total. Neither acid nor amide caused any increase in ethereal sulphate excretion.

Metabolism of p toluic acid and amide The results obtained with these compounds are summarized in Table 4. The ether soluble acid from the amide represents the extent of amide hydrolysis more nearly than in the case of *m* toluamide,

Table 3 *Metabolism of m toluic acid and amide in the rabbit*

Rabbit no	Percentage of dose excreted as	
	Ether soluble acid	Ester glucuronide
<i>m</i> Toluic acid (dose level 0.3 g/kg)		
134	104	33
134	96	40
182	90	23
185	—	25
191	—	28
216	100	28
229	75	27
229	81	29
231	100	27
233	87	9
233	103	18
234	—	26
244	—	30
Average	93	26
<i>m</i> Toluamide (dose level 0.3 g/kg)		
134	89	15
134	108	26
134	84	13
182	102	6
215	98	5
231	96	2
247	84	3
248	84	5
249	97	8
250	94	3
Average	94	9

Table 4 *Metabolism of p toluic acid and amide in the rabbit*

Rabbit no	Percentage of dose excreted as	
	Ether soluble acid	Ester glucuronide
<i>p</i> Toluic acid (dose level 0.3 g/kg)		
134	88	31
213	—	14
219	—	24
228	—	21
229	—	19
234	—	13
244	—	4
244	102	7
247	93	9
247	110	11
247	—	13
248	99	10
249	105	14
249	89	7
Average	98	14
<i>p</i> Toluamide (dose level 0.3 g/kg)		
182	70	5
215	67	3
220	—	2
221	88	5
221	57	4
231	71	2
234	92	4
Average	74	4

though here also a small proportion of the dose (at least 0.5%) is excreted with the carbamyl group intact and the methyl group oxidized to carboxyl (see p 49). The ester glucuronide formed is completely extracted by ether. Neither acid nor amide caused any increase in ethereal sulphate conjugation.

Hydrolysis of toluamides by rabbit liver extracts Solutions of the three amides (0.01204M) in phosphate buffer (pH 7.4) were incubated with rabbit-liver extracts prepared as previously described (Bray, James, Ryman & Thorpe, 1948) and the degree of hydrolysis measured by estimation of the NH_3 liberated (Bray, James, Raffan, Ryman & Thorpe, 1949). Table 5 summarizes the results obtained from three

Table 5 *Hydrolysis of toluamides and p nitrobenzamide by rabbit liver extracts at pH 7.4*

Amide	Average percentage hydrolysis by liver extract in	
	3 hr	20 hr
<i>o</i> Toluamide	2.5	3.0
<i>m</i> Toluamide	7.0	13.0
<i>p</i> Toluamide	19.0	48.5
<i>p</i> Nitrobenzamide	13.0	42.0

experiments with each isomer. In one of each of these, formal titrations gave results agreeing with the NH_3 values within the limits of experimental error. Values for *p* nitrobenzamide are also included in order to enable comparison to be made with the behaviour of an amide which has been shown to be completely hydrolysed in the intact animal (Bray, Thorpe & Wood, 1949).

Qualitative studies

Metabolites of *o* toluidic acid No metabolite of *o* toluidic acid could be isolated from the ether extract of the urine as collected from rabbits dosed with this acid. Continuous extraction of the acidified urine, however, yielded a brown syrup which gave strongly positive naphthoresorcinol and reducing (Benedict's) tests. The quantitative results recorded in Tables 1 and 2 suggested that this consisted primarily of ester glucuronide, but attempts to obtain this in a crystalline form were unsuccessful, probably owing to its instability. When a concentrated solution of the syrup in aqueous ethanol was allowed to evaporate at ordinary temperature in air, crystals of *o* toluidic acid were deposited, but whether from the decomposition of the glucuronide or because the acid was present as an individual excretion product could not be determined. It is possible that a small amount of *o* toluidic acid may be excreted unconjugated since in some experiments the ether soluble acid excretion was greater than the excretion of ester glucuronide (Table 1). Glycine conjugation, if it occurred at all, took place to only a slight extent since examination of the ether soluble material by toluene fractionation as described for the other isomers yielded no *o* toluidic acid.

We failed to isolate the glucuronide by the usual lead precipitation method which yielded only syrups from which crystalline products could not be obtained, even by means of ether extraction. Since the only product identified after hydrolysis of the ether extracts was *o* toluidic acid it is probable that the glucuronide is *o* toluidylglucuronide.

Metabolites of *o* toluamide The 24 hr urine of ten rabbits which had each received 0.5 g *o* toluamide was continuously

extracted with ether for 24 hr, first as collected (extract A) and then after acidification with 2N H_2SO_4 (extract B). The residue from the second extraction was hydrolysed by boiling and then continuously extracted with ether (extract C). Extract A was a clear yellow syrup which crystallized. Recrystallization from water gave white plates (m.p. 70°). During recrystallization they melted to form a pale yellow oil having an odour resembling that of the toluamides. The compound, which was nitrogen free, was identified as phthalide and gave no depression of melting point on mixing with an authentic specimen (m.p. 70°) (Found C, 71.3, H, 4.4. Calc. for $\text{C}_8\text{H}_6\text{O}_2$, C, 71.7, H, 4.5%). The total yield of phthalide was approx. 1 g.

Extract B was a dark brown syrup which slowly deposited crystals. The first crop (200 mg) was separated and after recrystallization from water had m.p. 116–118°. It appeared to be crude benzoic acid, giving no depression of melting point when mixed with the pure acid (m.p. 120°). The residual syrup finally crystallized almost completely. The solid material was separated on a porous tile and recrystallized from water (charcoal), and yielded 700 mg pure benzoic acid (m.p. 120°) (Found C, 69.2, H, 5.0. Calc. for $\text{C}_7\text{H}_6\text{O}_2$, C, 68.9, H, 4.9%). Its identity was confirmed by conversion to benzamide (m.p. 163°), which did not depress the m.p. 163° of an authentic specimen. No metabolite of *o* toluamide could be isolated from extract C.

Metabolites of *m* toluidic acid The ether soluble material from acidified *m* toluidic acid urine was fractionated by refluxing with toluene. The dark brown syrup was almost completely soluble in the hot solvent, but crystals and syrup were deposited on cooling. Recrystallization of this material from water (charcoal) gave needles (m.p. 140°), which were strongly acidic to Congo red and were identified as *m* toluidic acid (Found N, 7.0, equivalent (by titration) 190. Calc. for $\text{C}_9\text{H}_9\text{O}_3\text{N}$, N, 7.3%, equivalent 193. Gleditsch & Moeller (1889) gave m.p. 139°). The toluene filtrate obtained after separation of the crude toluidic acid was evaporated to dryness under reduced pressure and yielded a brown syrup. This, on treatment with dilute aqueous ethanol, gave needles which after recrystallization had m.p. 110° and were identical with an authentic specimen of *m* toluidic acid (mixed m.p. 110°). It was difficult to establish the yields of the two metabolites described, since it is certain that a proportion remained in the intractable syrupy residues. The amounts of toluidic and toluidic acids isolated were 1.0 and 0.5 g respectively from doses of *m* toluidic acid totalling 3 g. The only product isolated after hydrolysis of the syrupy residues was *m* toluidic acid.

Metabolites of *m* toluamide Extraction of *m* toluamide urine as collected gave no recognizable crystalline product, suggesting that little or none is excreted unmodified.

During continuous ether extraction of acidified *m* toluamide urine solid material separated from the ethereal solution. After 24 hr extraction this solid was separated, and, after exhaustive extraction with boiling toluene, recrystallized from aqueous ethanol. The product had m.p. 278° and on acid hydrolysis gave isophthalic acid (m.p. 334°). Analysis confirmed the identity of the metabolite with isophthalamic acid (*m* carbamylbenzoic acid) (Found N, 8.2, equivalent (by titration) 160. Calc. for $\text{C}_8\text{H}_7\text{O}_3\text{N}$, N, 8.5%, equivalent 165). Yield of isophthalamic acid 1.7 g from a total dose of 5 g *m* toluamide. The toluene soluble material appeared to be crude hippuric acid contaminated mainly with isophthalamic acid.

After removal of the crude isophthalamic acid from the original extract the ethereal filtrate was evaporated to

dryness and the residual syrup fractionated in the same way as the ether soluble material from *m* toluic acid urine. *m* Toluic and *m* toluic acids were thus isolated in yields of 0.3 and 1.8 g respectively from an original total dose of 5 g *m* toluamide. The final fraction from the *m* toluic acid mother liquors appeared to be crude acid similar to that extracted by toluene from the crude isophthalamic acid.

Metabolites of *p* toluic acid. The ether soluble material obtained by continuous extraction of acidified rabbit urine, corresponding to a dose of 3 g *p* toluic acid, was a light-brown syrup which crystallized almost completely on standing at room temperature. By trituration with water in the cold most of the reducing material was removed, leaving a residue which, after recrystallization from water (charcoal), gave white plates (m.p. 146–147°). These were extracted with boiling toluene and resolved into two fractions: (a) crystals (100 mg), readily soluble in toluene (m.p. 178–180°), which did not depress the m.p., 179°, of an authentic specimen of *p* toluic acid, and (b) crystals (2 g) only slightly soluble in toluene (m.p. 163°). These were strongly acidic to Congo red and proved to be *p* toluic acid. (Found: N, 7.3, equivalent (by titration) 189. Calc. for $C_{10}H_{11}O_3N$, N, 7.3%, equivalent 193. Gleditsch & Moeller (1889) found m.p. 161–162°.)

The aqueous trituration (above) was extracted by shaking with ether. Evaporation of the ether from the extract yielded only a small amount of *p* toluic acid. The residue after extraction reduced Benedict reagent, but we were not able to isolate *p* toluylglucuronic acid. Attempts to obtain it by the normal lead precipitation procedure were also unsuccessful.

Metabolites of *p* toluamide. The chief metabolite of *p* toluamide is *p* toluic acid which was isolated (2.5 g corresponding to the administration of 4 g amide), together with small amounts of *p* toluic acid (120 mg), by the method described above. The amounts obtained were similar to those isolated from *p* toluic acid urine. It was also observed that a small amount of material was much less soluble in water than were toluic or toluic acids. This was separated and recrystallized from aqueous ethanol. It was acid to Congo red and did not melt, but sublimed at about 300°. These properties were consistent with those of terephthalamic acid (*p* carbamylbenzoic acid). (Found: N, 8.1%. Calc. for $C_8H_7O_3N$, N, 8.5%.) Yield 20 mg.

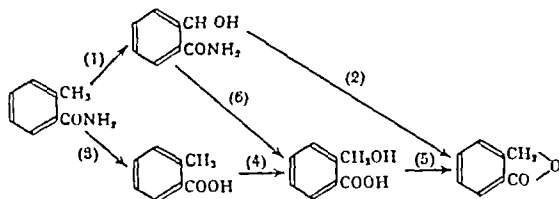
Extraction of *p* toluamide urine as collected gave needles melting at 170° which were not *p*-toluamide or *p* toluic acid. The amount isolated (5 mg) was not sufficient for identification.

DISCUSSION

In an earlier paper of this series (Bray, Ryman & Thorpe, 1948) we discussed the metabolism of various aromatic compounds from the point of view of the presence in their molecules of groupings which can be directly conjugated and of groupings which can be modified so that conjugation can then take place. The metabolism of the toluamides is of particular interest in this connexion. These compounds contain no directly conjugable groups, but both the methyl and carbamyl groups are potential centres for conjugation inasmuch as they may be converted to carboxyl groups, by oxidation and hydrolysis respectively. The three isomers show a gradation in metabolic behaviour. In the *para*

isomer the carbamyl group is largely hydrolysed and the excretion products are very similar to those of the corresponding acid. Hydrolysis is not quite complete, however, since a small amount of terephthalamic acid is formed, indicating that oxidation of the other potential centre for conjugation does occur. The terephthalamic acid isolated appears to have been excreted unconjugated. In *m* toluamide the extent of the hydrolytic and oxidative reactions is markedly different from that in the *para* isomer. Nearly half the dose is excreted as isophthalamic acid, and the remainder as derivatives of *m* toluic acid. Since both terephthalamic and isophthalamic acid are excreted unconjugated, it appears that potential centres for conjugation may be converted into actual centres even if conjugation cannot or does not then take place.

The excretion product of *o* toluamide isolated merits individual consideration. Phthalide may arise from *o* toluamide by means of the following reactions:



The fact that the studies *in vitro* suggested that the carbamyl group in *o* toluamide is biologically stable may be interpreted as evidence against a metabolic pathway involving reactions (3), (4) and (5), as may also the fact that phthalide could not be isolated from *o* toluic acid urine. Failure to isolate *o* hydroxymethylbenzoic acid should not be regarded as final evidence against reaction (5), owing to the ease with which this compound is converted to phthalide. It is unfortunate that *o* hydroxymethylbenzamide has not been prepared, since its properties might determine whether phthalide could be formed directly by reaction (2), or only through *o* hydroxymethylbenzoic acid (reactions (6) and (5)). It thus appears that the first metabolic reaction which *o* toluamide undergoes in the rabbit is oxidation of the methyl group. The resulting primary alcohol is however labile, undergoing, directly or indirectly, ring closure to form phthalide. So far as we are aware there is only one cited example of the isolation of this intermediate stage in the oxidation *in vivo* of a methyl group attached to the aromatic nucleus. This is the isolation of *o* nitrobenzyl alcohol as a metabolite of *o* nitrotoluene (Jaffé, 1878–9). It is often difficult to isolate intermediate products in biological reactions of this type and lactonization such as occurs in the formation of phthalide may provide a means of 'fixing' and identifying the intermediate products in other oxidations. We are

examining some other alkyl derivatives of benzamide with this end in view

It is clear from the *in vivo* experiments that *m* and *p* toluamides can act as substrates for two different enzyme systems, one oxidizing the methyl group and another hydrolysing the carbamyl group. The results of *in vitro* experiments with liver extract recorded in Table 5 were obtained by estimation of ammonia and thus represent the hydrolysis of the carbamyl group only. Since corresponding results were obtained by formol titration, it seems probable that the principal, if not the only, reaction occurring in digests with liver extracts is hydrolysis of the carbamyl group. The use of extracts appears, in effect, to isolate one of the enzyme systems.

In view of the fact that only phthalide was isolated as a metabolite of *o* toluamide it is difficult to comment upon the analytical results obtained (Table 1). The significance of the reducing values has already been discussed. The benzoic acid isolated from acidified but unhydrolysed *o* toluamide urine is presumably a normal metabolite of the rabbit, though it is not usually isolated in such comparatively large amounts. If *o* toluamide inhibits glycine conjugation it would not be expected that the amount of ether-soluble acid would differ greatly from the normal amount, since *in vitro* experiments suggest that the carbamyl group of *o* toluamide is stable. The analytical results, however, suggest that extra ether-soluble acid is usually excreted, although the amount is variable. We have not yet been able to determine the nature of this extra acid.

SUMMARY

1 The metabolism of the toluic acids and their amides in the rabbit has been studied both quantitatively and qualitatively.

2 The acids are excreted as conjugates of glycine or glucuronic acid, the latter being ether-soluble. Small amounts are excreted unchanged.

With the amides, the methyl group may be oxidized or the carbamyl group hydrolysed (or both), and the resulting carboxylic acids excreted free or conjugated as above. With the possible exception of *o* toluamide, there is no evidence of ethereal sulphate formation.

3 The percentages of the dose excreted as ether-soluble acid were: acids—*ortho*, 77%, *meta*, 93% and *para*, 98%; amides—*meta*, 94% and *para*, 74%. These values include the ether-soluble ester glucuronides.

4 *m* and *p* Toluic acids are excreted unchanged (17 and 3% respectively isolated), and conjugated with glucuronic acid (26 and 14%), and with glycine (23 and 46% isolated). *o* Toluic acid is conjugated with glucuronic acid, but glycine conjugation was not observed.

5 After dosage with *m* and *p* toluamides, toluic (6 and 3% respectively), toluic (25 and 43%), and isophthalamic (28%) or terephthalamic (0.4%) acids were isolated. Glucuronide excretion accounted for 9 and 4% respectively.

6 The only metabolite of *o* toluamide isolated was phthalide (20% of the dose), which probably arose from *o* hydroxymethylbenzoic acid or amide. Consistent analytical results could not be obtained.

7 Rabbit liver extracts hydrolyse *o*, *m*- and *p* toluamides to the extent of 3, 13 and 49% respectively.

The authors are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this investigation, and to Dr Sybil P. James and Miss M. R. Wasdell for carrying out the enzyme experiments. The microanalyses were carried out by Drs Weiler and Strauss, Oxford.

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Properties of the Acid Phosphatases of Erythrocytes and of the Human Prostate Gland

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(Received 25 February 1949)

There is much evidence indicating the non identity of acid phosphatases of different origin. Davies (1934) has shown that the acid phosphatase of the red cell differs from that of spleen in that the former hydrolyses α glycerophosphate much more readily than the β compound, while with the spleen enzyme β glycerophosphate is hydrolysed more quickly. Kutscher & Worner (1936) described the irreversible inactivation of the prostatic acid phosphatase by certain narcotics, including alcohols. This was used by Herbert (1944, 1945, 1946) for the identification and determination of the prostatic enzyme in blood serum, whose normal acid phosphatase is hardly affected by ethanol treatment. King, Wood & Delory (1945), on the other hand, found the acid phosphatases of prostate and red cells to be similar in many respects, including easy destruction by ethanol, but formaldehyde and L tartrate (Abul-Fadl & King, 1947, 1948*a, b*) sharply distinguish the two enzymes by complete inactivation of the one or the other. In the present investigation the question of the identity of these two acid phosphatases is further dealt with. For this purpose, the rates of hydrolysis of phenyl phosphate by each enzyme at different pH values were determined, together with the relative rates of hydrolysis of α and β glycerophosphates. This was followed by studies of the effects of organic and inorganic substances belonging to different groups of a possible activating or inhibiting nature. The nature of the enzymes is discussed in the light of these experiments.

EXPERIMENTAL

Enzyme solutions

Normal fresh human prostate glands were decapsulated, cut into fine pieces and ground with sand in a mortar with 5 times their weight of 0.9% NaCl solution. A few drops of toluene were added and the mixture was left at room temperature to autolyse for 2-3 days. The mixture was then filtered and highly diluted (e.g. 1000-5000 times) before determining its activity.

Freshly drawn samples of blood from man, ox, sheep, pig, rabbit, rat and guinea pig were centrifuged, the plasma with the top layer of white cells separated, and the red cells twice washed with 0.9% NaCl by spinning and decantation. Washed red cells (1 ml.) were lysed in 9 ml. water and the solution centrifuged. The clear supernatant was used immediately for determining the enzyme activity.

Determination of pH hydrolysis curves

Acetate buffer solutions were prepared to cover the range of pH 3.6-6.0. The pH of each solution was determined by the indicator method and by the glass electrode (Muirhead) after mixing with an equal volume of the 0.02M disodium phenyl phosphate substrate solution and warming the mixture to 37° in a water bath. The hydrolysis period was exactly 30 min. at 37°, when the liberated phosphorus or phenol was determined.

Activating and inhibiting substances

Metallic ions, acid radicals and amino acids. The effects of the following substances on the rates of enzymic hydrolysis of phenyl phosphate by the prostatic and the red cell acid phosphatases have been investigated. Ca^{++} , Co^{++} , Cr^{+++} , Cu^{++} , Fe^{+++} , Mn^{++} and Zn^{++} , arsenate, citrate, cyanide, formate, fluoride, oxalate, salicylate, tartrate and tauroglycocholate, glycine, alanine, cysteine and glutathione. Solutions of the pure substances (A.R. whenever possible) were prepared so that 1 ml. of each solution, when mixed with buffer, substrate and enzyme solutions, would furnish the appropriate concentration of the substance in the final mixture. Acetate or citrate buffers of pH 5.0 were used. The liberated phenol was determined as an index of activity, except when there was a direct interference by certain metals or other substances with the method of phenol estimation. In such cases the liberated phosphate was determined either by the King (1932) method or by the Allen (1940) amidol method, and acetate was used instead of citrate buffer which interferes with the determination of free phosphate.

Iodoacetic acid. Neutral solutions containing 5-10 mg. of iodoacetate were mixed with the enzyme solutions and the activities were determined with and without previous incubation.

Stilboestrol (4,4'-dihydroxy $\alpha\beta$ -diethylstilbene). Tablets containing 5 mg. stilboestrol were ground in a mortar and thoroughly extracted with dry chloroform. The clear filtered chloroform extract was adjusted to a concentration of 1 mg. stilboestrol/ml.

The effect of this substance on prostatic and red cell phosphatases was studied in two ways. (a) by incubating the enzyme solution with stilboestrol for 1 hr. at room temperature, and comparing its activity with that of untreated solution, which was similarly incubated. For this purpose 5 ml. stilboestrol solution in chloroform (1 mg./ml.) were pipetted into a 100 ml. measuring flask and evaporated by gentle warming in a water bath. The flask was then filled with the enzyme solution, and kept at room temperature for 1 hr. with gentle rotation to assure saturation and equal distribution of the stilboestrol. (b) By including 0.5 mg. stilboestrol in the buffer substrate enzyme mixture.

In every case, chloroform was completely eliminated before mixing with the enzyme. This is especially important in the case of the prostatic phosphatase which is sensitive to this substance. Vigorous shaking was avoided, since the prostatic enzyme is rapidly inactivated, especially in dilute solutions.

RESULTS

The pH hydrolysis curve indicates the presence of two acid phosphatases in the red cells of man and other animals, a 'first acid enzyme' with optimum

temperature for a few hours. In some species the two optima lie well apart from each other and are fairly sharp (e.g. human, rabbit, ox and sheep, Fig 1). In other species, however, the two optima are so close to each other, and that of the second enzyme (of higher pH optimum) is so broad that they are difficult to determine (e.g. pig and rat, Fig 2).

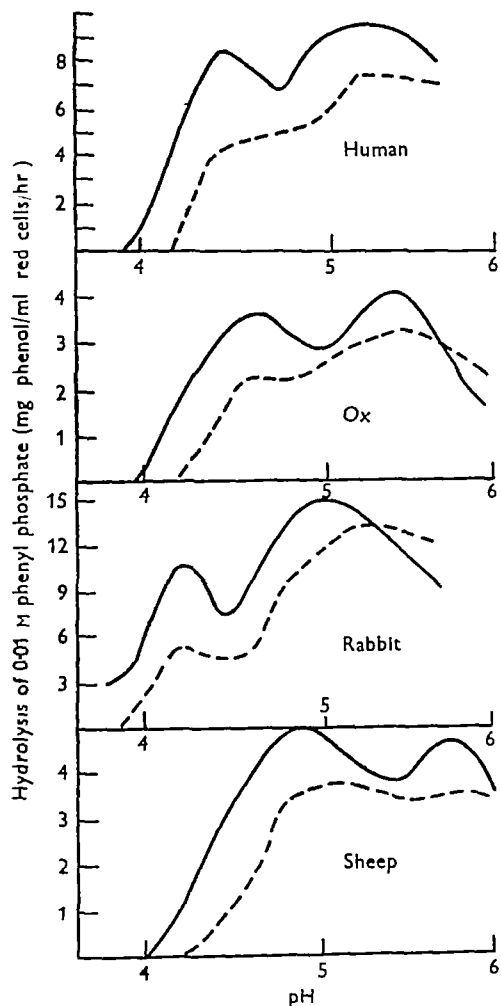


Fig 1 pH-Hydrolysis curves for the acid phosphatases of the red cells. Solid line without added Mg^{++} , broken line, with added 0.01 M Mg^{++} .

pH of 4.3–4.8 and a 'second acid enzyme' optimally active at pH 5.0–5.7. The optimum for each enzyme was found to vary a little, not only from one species to another, but even in different individuals of the same animal species. The first acid enzyme is very labile and can no longer be detected in enzyme solutions which have been left to stand at room

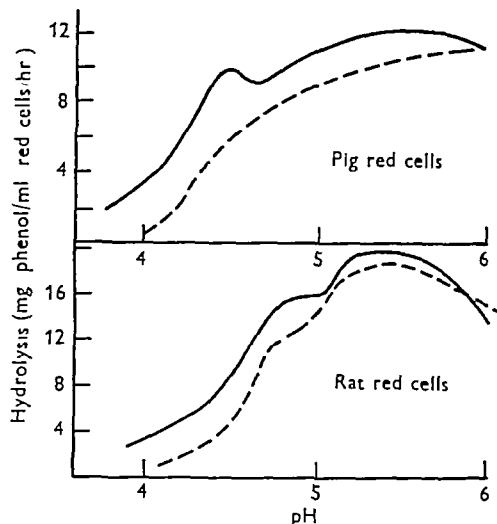


Fig 2 pH Hydrolysis curves of 0.01 M phenyl phosphate by red cell acid phosphatases. Solid line, without added Mg^{++} , broken line, with added 0.01 M Mg^{++} .

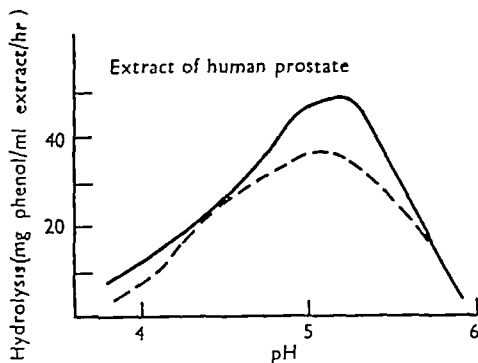


Fig 3 pH Hydrolysis curve of 0.01 M phenyl phosphate by prostatic acid phosphatase. Solid line, without added Mg^{++} , broken line, with added 0.01 M Mg^{++} .

Magnesium inhibits the phosphatases of the red cells especially in the more acid range. In some cases, where the enzyme activity was still marked at pH 6.0, magnesium was either without effect or showed slight activation, but in every case there was pronounced inhibition at the optimal pH.

Fig 3 shows the shape of the pH-hydrolysis curve of phenyl phosphate by prostatic acid phosphatase. There is only one clearly defined optimum, at pH 5.0–5.2. Magnesium, at different pH values, was

either without effect or produced inhibition only near the optimum. The absence of marked inhibition at the more acid pH, which was very pronounced in the case of the phosphatases of the red cells, confirms the homogeneity of the prostatic enzyme.

Table 1 shows the relative rates of hydrolysis of α and β glycerophosphates and phenyl phosphate by the acid phosphatases of the prostate and red cells. β Glycerophosphate is very slowly hydrolysed by the red cell enzyme. The α isomer is more quickly hydrolysed and phenyl phosphate much more rapidly. The very low activity of red cell phosphatases against β glycerophosphate has been repeatedly confirmed with red cells from different

β glycerophosphates, but it seems to inhibit very slightly the hydrolysis of phenyl phosphate (though not as much as it does with the red cell enzyme).

Table 2 shows the effect of certain metallic ions on the acid phosphatases of plasma, prostate and red cells. In acetate buffer calcium inhibits the red cell enzyme slightly and the prostatic enzyme more strongly. (In citrate buffer, however, these inhibitory effects completely disappear owing to the formation of calcium complexes with the citrate.) Chromium, cobalt, manganese and zinc all have variable inhibitory effects on prostatic and red cell phosphatases, and none was found to exhibit the slightest activating property.

Table 1 *Relative rates of hydrolysis of α - and β glycerophosphates and phenyl phosphate by the acid phosphatases of the prostate and red cells*

(Acetate buffer pH 5, 30 min at 37°)

Enzyme solution	Hydrolysis (mg P/30 min /100 ml enzyme solution)											
	0.02M β Glycerophosphate				0.02M α Glycerophosphate				0.005M Phenyl phosphate			
	Without			Mg	Without			Mg	Without			Mg
	Mg	0.003M Mg	0.01M Mg		Mg	0.003M Mg	0.01M Mg		Mg	0.003M Mg	0.01M Mg	
Red cells (human)	0.2	0.25	0.3	11	11	11	55	—	48			
Red cells (human)	0.2	0.2	0.25	9.2	9.2	—	43	37	—			
Prostate in normal saline	29	29	30	28	28	—	53	—	50			

Table 2 *Effect of metallic ions on the acid phosphatases of plasma, prostate and red cells*

(Sodium phenyl phosphate substrate, acetate buffer, pH 5, 30 min at 37° Activity expressed as 'units', i.e. mg P/30 min /100 ml enzyme solution)

Enzyme solution	Control enzyme without inhibitor (units/100 ml)	Percentage inhibition					
		Calcium (0.01M)	Chromium (0.01M)	Cobalt (0.01M)	Manganese (0.01M)	Nickel (0.01M)	Zinc (0.001M)
Red cells (human)	80	14	25	0	15	—	20
Red cells (human)	47	17	17	4	15	23	23
Red cells (rat)	88	10	20	—	17	—	—
Plasma (normal)	4.2	0	—	0	10	28	0
Prostate in normal saline	50	40	8	0	4	11	40
Seminal fluid	160	50	9	—	7	21	52
Prostate in normal plasma	10.0	16	—	0	0	4	12

sources and with different dilutions of enzyme, which were incubated with this substrate and acetate buffers at pH values from 3.6 to 6.6 for various lengths of time. (The glycerophosphate was a crystalline sample (Boots Pure Drug Co. Ltd.), and was rapidly hydrolysed by the prostatic enzyme, as well as by several alkaline phosphatases.) Magnesium at different concentrations activates very slightly the hydrolysis of β glycerophosphate while practically not affecting that of the α isomer. It invariably inhibits the splitting of the phenyl ester.

The prostatic enzyme, on the other hand, hydrolyses the β glycerophosphate almost at the same rate as the α -ester, while the phenyl ester is hydrolysed about twice as fast. The effect of magnesium (0.003 and 0.01M) is almost negligible with the α and

Table 3 shows the effect of copper and iron on the acid phosphatases of the prostate and red cells. That the nature of the buffer is of considerable importance in the study of the effect of metals is also clearly shown. Copper in concentrations as low as 0.0002M is a strong inhibitor of the red cell phosphatases (about 90% inhibition) in either acetate or citrate buffer media. With the prostatic enzyme copper in acetate causes slight inhibition (about 8% with 0.0002M CuSO_4), with citrate buffer, on the other hand, a marked activation is noticed with 0.001 and 0.0005M copper sulphate.

In an acetate buffer the iron present is in the form of a colloidal basic iron acetate. This causes a serious inhibition of the prostatic enzyme (80–90%) and a less marked inhibition of the red cell phosphatases.

Table 3 *Effect of copper and iron on the acid phosphatases of prostate and red cells in presence of acetate and citrate buffer solutions*

(Disodium phenyl phosphate substrate, 30 min at 37° Activity expressed as mg P/30 min /100 ml enzyme solution)

Enzyme solution	Enzyme activity without inhibitor (units/100 ml)	Percentage inhibition in presence of copper			Percentage inhibition in presence of iron	
		0 001 M	0 0005 M	0 0002 M	0 001 M	0 0005 M
		Acetate buffer pH 5 0				
Red cells (human)	53	96	96	95	25	9
Red cells (human)	47	100	—	—	25	9
Red cells (human)	80	90	88	85	18	5
Prostate in normal saline	50	22	10	—	89	—
Prostate in plasma	60	42	18	8	78	78
Citrate buffer pH 5 0						
Red cells (human)	47	99	94	90	6	0
Prostate	145	(15 A.)*	(8 A.)*	0	0	0

* A = Activation.

Table 4 *Effect of glycine, alanine and cysteine on the acid phosphatases of prostate and red cells*

(Acetate buffer pH 5, 0.005M phenyl phosphate substrate, 30 min at 37°)

Enzyme solution	Control (nothing added)	Hydrolysis (mg P/hr /100 ml. enzyme solution)											
		Glycine (M)				Alanine (M)				Cysteine (M)			
		0 02	0 01	0 005	0 0001	0 02	0 01	0 005	0 0001	0 02	0 01	0 005	0 001
Red cells (human)	22	23	23	22	22	—	—	—	—	—	—	—	—
Red cells (rat)	40	—	—	—	—	—	—	—	—	36	40	42	—
Red cells (human)	28	28	29	29	29	28	28	28	30	27	30	30	30
Red cells (human)*	29	29	29	30	29	29	30	30	30	27	29	31	30
Prostate in normal saline	41	36	37	40	42	37	39	40	42	42	45	43	43
Prostate in normal saline*	40	36	37	39	39	—	—	—	—	37	43	44	42
Seminal fluid in normal saline	29	—	—	—	—	—	—	—	—	26	31	—	34

* After 3 days' dialysis in cellophan bag at 6° against running distilled water

(10–25 %) In citrate buffer medium, however, the pronounced inhibitory action on the prostatic enzyme is completely annulled, while the inhibition of the red cell enzyme is still marked with 0 001M-concentration, but is abolished when the concentration is reduced to 0 0005M

Table 4 shows the effect of glycine, alanine, and cysteine on the phosphatases of prostate and red cells The concentrations of glycine and alanine, which have been reported to activate the alkaline phosphatases, do not exhibit such an effect on the acid enzymes Higher concentrations of these amino acids have no effect on the red cell enzymes, but slightly inhibit the prostatic phosphatase Cysteine in 0 02M concentration produces a slight inhibition with red cell and prostatic enzymes, 0 01M concentration is either without effect or slightly activates both enzymes After prolonged dialysis of acid phosphatases, there was still no change in response towards amino acids

Table 5 shows the effect of cysteine and glutathione on the copper inhibited phosphatases of the red cells When cysteine or glutathione was added, together with copper, to the enzyme solution, the latter did not inhibit the enzyme markedly But if copper was

Table 5 *Effect of cysteine and glutathione on the copper inhibited phosphatases of the red cells*

(Acetate buffer, pH 5, 0.005M phenyl phosphate substrate, 1 hr at 37°, two experiments, A and B)

	Hydrolysis (mg P/hr /100 ml enzyme solution)	
	A	B
Red cell solution alone	45	19
Red cell solution with 0 01M cysteine	46	17
Red cell solution with 0 01M glutathione	46	18
Red cell solution with 0 0005M CuSO ₄	8	2
Red cell solution with 0 02M cysteine + 0 0005M CuSO ₄ both added at the same time	36	15
Red cell solution incubated with 0 0002M CuSO ₄ for 15 min, activity determined in presence of 0 02M cysteine	8	2
Same experiment repeated, substituting cysteine by 0.02M glutathione	8	2

allowed to react with the enzyme first, the enzyme was inhibited, and it did not seem possible to reverse this inhibition with excess of either cysteine or glutathione

Table 6 shows the effect of certain acid radicals. Arsenate strongly inhibited (about 80%) the red-cell phosphatases and also inhibited by about 70%

acids (hydroxy, ketonic, dicarboxylic and ethylenic) was tested. None was even nearly as effective as tartaric acid.

On trying the effect of the different stereoisomers of tartaric acid, however, the inhibitory action was found to be exhibited only by the L* and the DL racemic forms, not by the D or the meso form.

Table 6 *Effect of acid radicals on the acid phosphatases of plasma, prostate and red cells*

(Acetate buffer, pH 5, phenyl phosphate substrate, liberated phosphate determined after 30 min. at 37°. The figures represent the average percentage of activation (+) or inhibition (-) effected in several experiments.)

Acid radical (0.01M)	Activation or inhibition				
	Normal plasma (%)	Human red cells in water (%)	Prostate in normal saline (%)	Prostate in normal plasma (%)	Seminal fluid in normal saline (%)
Arsenate	-72	-80	-66	-70	-66
Citrate	0	+5	+8	+5	+6
Cyanide	0	+8	+12	+10	+15
Fluoride	-30	-8	-96	-96	-97
Formate	0	0	0	0	0
Oxalate	-30	-27	-22	-25	-25
Salicylate	0	0	0	0	0
Tartrate (L)	0	0	-94	-95	-93
Tauroglycocholate	0	-77	-76	-85	-80

the enzymes of plasma and prostate. There is a slight activation of the red cell and prostatic enzymes by the citrate radical, while the formate and the salicylate radicals are without any effect. Fluoride in 0.01M concentration has comparatively little effect (8-10% inhibition) on the red cell phosphatases, while exhibiting more inhibition (28%) of plasma phosphatase, and very marked (more than 95%) inhibition of the prostatic enzyme. Cyanide (0.01M) was found to be either without effect or to have mild activating properties (up to 15%) on both red cell and prostatic acid phosphatases. Oxalate (0.01M) inhibited the plasma, prostatic and red cell phosphatases nearly to the same extent (about 25%). Bile salts in the form of sodium tauroglycocholate (used in a final concentration of 0.24% in buffer substrate-enzyme mixture) strongly inhibited both prostatic and red cell enzymes (70-90%). The normal plasma phosphatase, on the other hand, did not seem to be affected. Samples of pathological plasma were also examined. Some of them showed slightly raised acid phosphatase values in cases of liver disease, and all were found not to be affected by this bile salt.

Tartrate, while having no effect whatever on the acid phosphatase of either red cells or normal plasma, was found to be a very strong inhibitor of the prostatic enzyme (more than 90% inhibition in 0.01M concentration).

Table 7 shows the effect of certain organic acid radicals, which are structurally related to tartaric acid, on the phosphatases of plasma, prostate and red cells. A series of aliphatic and aromatic organic

Dextrorotatory substances like dextrose have been tried also, as well as glycerol, pinacol, sorbitol,

Table 7 *Effect of organic acid radicals related to tartaric on the acid phosphatases of plasma, prostate and red cells*

(Citrate buffer, pH 5, 30 min. at 37°, liberated phenol was determined, except in the case of certain acids marked †, where the free phosphate was determined instead, and acetate buffer pH 5 was used in place of citrate. The acid solutions were adjusted to pH 5 before mixing with the buffer substrate mixture. The figures represent the average percentage activation (+), or inhibition (-) effected.)

Acid (0.01M unless otherwise stated)	Plasma	Red cells	Prostate
Lactic	0	0	0
Pyruvate†	+20	0	0
Malic	0	+8	+12
Maleate†	0	0	0
Malonic	0	0	0
Mucic	0	0	0
Fumaric	0	0	0
Succinic	0	0	0
Tartaric	0	0	0
D-Tartaric	0	0	0
DL-Tartaric	0	0	-92
meso-Tartaric	0	0	0
L* Tartaric	0	0	-94
L Tartaric (0.02M)	0	0	-95
L-Tartaric (0.005M)	0	0	-90
L Tartaric (0.002M)	0	0	-75
L-Tartaric (0.001M)	0	0	-70

xylose and laevulose, but none produced an effect similar to L-tartrate.

* The L-form is the common dextrorotatory acid.

Table 8 shows that a quantitative determination of red cell acid phosphatase, when present in admixture with the prostatic enzyme in serum or plasma, is a practical procedure, and the figures found are reasonably close to the calculated values. It is also possible by this means to demonstrate the presence of a tissue phosphatase, as distinct from the plasma and red cell enzymes, in blood or in mixtures containing blood (Tables 8 and 11)

Table 8 Differential determination of red cell and prostatic phosphatases in serum by means of L tartrate

	Phosphatase (units/100 ml solution)			
	Found		Calculated	
	Without tartrate	With 0.01M L tartrate	Red cell	Total
Normal serum	30	30	—	—
A, Prostate extract in normal serum	108	36	—	—
B, Red cell solution in normal serum	66	66	—	—
A and B mixed in the following proportions				
1 1	87	34	33	87
1 2	79	45	44	80
2 1	97	25	22	94

Table 9 shows that the inhibition of the prostatic enzyme by 0.005M L tartrate is reversible, and that the enzyme recovers its activity on dialysis. A control consisting of the enzyme without tartrate was set up, and similarly dialysed to safeguard against any extraneous change in the enzyme, either through dialysis or through incubation. Recovery was more rapid at room temperature than at 0°, but some inactivation results from incubation at room temperature.

Table 9 Reversal of L tartrate inhibition of prostatic phosphatase by dialysis

	Activity (units/100 ml enzyme solution)		
	Control (without tartrate)	Test (0.005M tartrate)	Inhibition (%)
Start	120	15	88
After 24 hr dialysis in cellophan at 5°	120	43	64
After further 24 hr dia- lysis at room temperature	75	64	15

Fig. 4 shows the effect of L tartrate on the acid phosphatases of red cells, prostate, liver and spleen at different pH values. Strong inhibition occurs with the last three enzyme preparations. With the

red cells inhibition is only detectable in the more acid part of the curve, while at the second optimum pH there is either no effect or slight activation. The alkaline phosphatases are slightly activated by L tartrate.

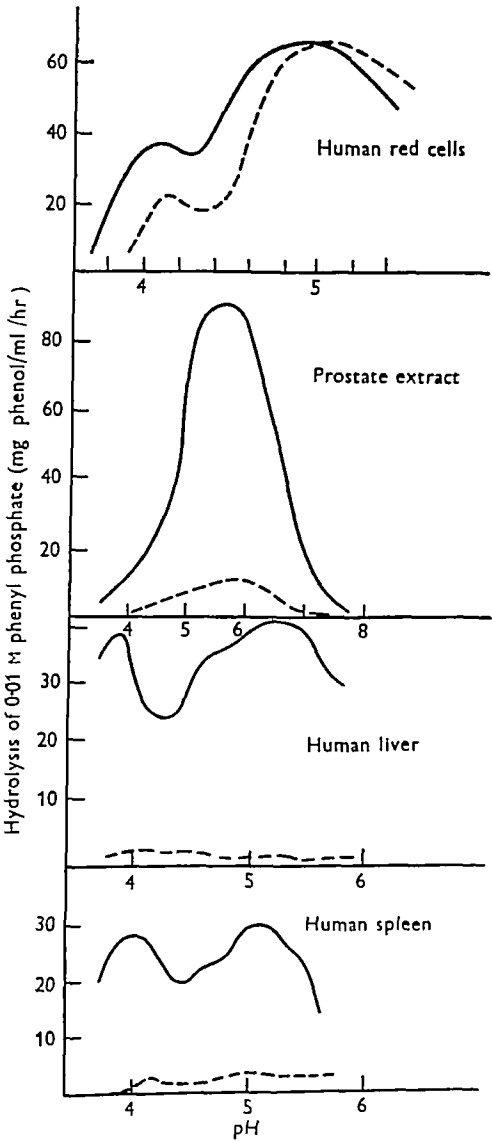


Fig. 4 Effect of L-tartrate on the hydrolysis of phenyl phosphate by acid phosphatases. Solid line, without added L-tartrate, broken line, with added 0.01 M L-tartrate.

Table 10 shows that the inhibitory effect of 10% acetic acid on the prostatic and the red cell enzymes is quite evident even without previous incubation of this substance with the enzyme. Both enzymes are inhibited nearly to the same extent.

Table 11 shows the effect of certain other organic substances on acid phosphatases. Incubation with different alcohols, especially the lower homologues,

Table 10 *Effect of iodoacetic acid on the acid phosphatases of prostate and red cells*

(Acetate buffer, pH 5, 0.005M phenyl phosphate, 30 min at 37° Iodoacetic acid solution neutralized before mixing with the enzyme Activity mg P/30 min /100 ml enzyme solution

Enzyme	Control without inhibitor (mg P)	Iodoacetic acid		
		Amount added (mg)	Activity (mg P)	Inhibition (%)
Red cell (human)	39	5	16	59
Red cell solution	39	5	9	77
incubated with inhibitor for 30 min at 6° at pH 7	39	10	4	90
Prostate extract in normal saline	150	5	62	59
	150	10	40	73
Prostate extract incubated with inhibitor for 30 min. at 6° at pH 7	150	5	26	83

inhibits the acid phosphatases of bile, prostate, red cells and kidney, and slightly affects that of normal plasma Acetone produces a similar effect Formaldehyde, in 0.5% concentration in the buffer substrate mixture, completely inhibits the red cell phosphatases and partially destroys the plasma, liver,

Table 12 *Summary of the effect of different treatments on the acid phosphatases of prostate and red cells*

Treatment	Prostatic acid phosphatase	Red cell acid phosphatase
Alcohol incubation	Inhibition 90-100%	Inhibition 70-80%
Copper (0.0002M)		
Acetate buffer	Inhibition 8%	Inhibition 96%
Citrate buffer	No effect	Inhibition 90%
Cyanide	Slight activation	Slight activation
Cysteine	Slight activation	Slight activation
Formaldehyde (0.5%)	No effect	Inhibition 100%
Fluoride (0.01M)	Inhibition 97%	Inhibition 10%
Hydrolysis of α and β glycerophosphoric esters	β slightly $> \alpha$	$\alpha > \beta$
Iron (0.0005M)		
Acetate buffer	Inhibition 80%	Inhibition 9%
Citrate buffer	No effect (or slight activation)	No effect
Magnesium (0.01M)	No effect (or slight inhibition)	Inhibition
Manganese	Slight inhibition	Slight inhibition

DISCUSSION

Roche, Thoaï & Baudoin (1942) reported the presence of two phosphomonoesterases in the red cells of the ox and rat, optimally active at pH 4.6-4.8 and at pH 5-5.5 respectively They added that the first enzyme is very unstable and is strongly inhibited by

Table 11 *Effect of certain organic substances on acid phosphatases*

(Citrate buffer pH 5, phenyl phosphate In the case of alcohols and acetone the enzyme was previously incubated with two fifths its volume of the corresponding alcohol or acetone before determining its activity The other substances were included in the buffer substrate mixture during hydrolysis The figures represent the average percentage inhibition)

Enzyme	Inhibition (%)						
	Alcohol			Acetone	Formaldehyde (0.5%)	L. Tartrate (0.01M)	Stilboestrol
	Methanol	Ethanol	Butanol				
Red cells	60	75	80	70	100	0	0
Prostate in normal saline	65	80	90	100	0	95	0
Prostate in normal plasma	100	100	100	100	0	95	0
Normal plasma	10	10	15	30	40	0	0
Liver							
Human (adult)	—	0	—	0	35	70	0
Human (new born)	—	0	—	—	20	70	0
Bile	—	80	—	—	35	0	—
Kidney							
Human (adult)	—	40	—	—	40	80	0
Human (new born)	—	50	—	—	20	85	0
Spleen							
Human (adult)	—	0	—	—	40	70	0

kidney and spleen enzymes (20-60%) The prostatic enzyme, on the other hand, is not affected to any extent under these conditions of treatment Stilboestrol is shown to have no detectable effect on any of these tissue acid phosphatases

Table 12 summarizes some of the above results

magnesium and manganese, while the second acid enzyme is relatively stable and is activated by these two metals The present work has confirmed these authors' claims as regards the presence of two acid phosphatases in the red cells of humans as well as other animals The first enzyme has been found to be

very unstable and unless the blood is very fresh it may escape detection. It is much more strongly inhibited by magnesium than the second acid enzyme, and is sensitive to L tartrate which does not affect the latter.

On the other hand, the presence of only one optimum in the pH hydrolysis curve of phenyl phosphate by the prostatic enzyme has been proved.

The acid phosphatase of mammalian erythrocytes has been reported to be activated by magnesium (Jenner & Kay, 1931, Davies, 1934, Roche, 1931, Roche *et al* 1942). Behrendt (1943), on the other hand, reported magnesium to be without effect on the phosphatases of human erythrocytes. Kutscher & Worner (1936) also found that magnesium has no effect on the prostatic enzyme. In the present investigation, magnesium has been found to activate slightly the hydrolysis of β glycerophosphate by the red cell enzyme but to exert no effect on the hydrolysis of the α -ester. On the other hand, it definitely inhibits the splitting of the phenyl phosphate. The prostatic enzyme, however, is either not affected by magnesium or only slightly inhibited. The fact that no conclusive evidence has been obtained concerning the activation of the prostatic or the red cell phosphatases by metals, especially magnesium even after prolonged dialysis, suggests that these acid enzymes do not consist of the metallo-protein structure which has been suggested for the alkaline phosphatases. It might be possible that they are saturated with the metal which is strongly bound to their protein molecule and which cannot be split before the complete denaturation of the protein component of the enzyme. Against this stands the fact that the acid phosphatases are either not affected, or are slightly activated, by cyanides, which strongly and irreversibly inhibit the alkaline enzymes presumably by combination with their essential metal. The inhibition of acid phosphatases by fluorides could not be accounted for by combination of the latter with a metal, since fluorides have either no effect or slightly activate the alkaline phosphatases. The inhibition of acid phosphatases by fluoride can be more reasonably considered as competitive, since Belfanti, Contardi & Ercoli (1935) and Lundquist (1946*a, b*) reported its reversibility by dialysis. The strong inhibitory effect of fluoride on the prostatic enzyme, as compared with its relatively mild action on the red-cell enzyme, is worthy of note.

The high sensitivity of the red cell acid phosphatases to minute amounts of copper may be taken as evidence for the necessity of thiol groups for the activity of these enzymes. These groups, on the other hand, seem to be not so important for the activity of the prostatic enzyme, since the latter is slightly activated by 0.001M copper sulphate in citrate buffer. The same explanation can be applied to the

peculiar action of formaldehyde, which under certain conditions completely and irreversibly inhibits the red cell phosphatases while not affecting the prostatic enzyme. (The action of formaldehyde can also be explained on the basis of the necessity of an amino group, which Gould (1944) claims to be essential for complete activity of the intestinal alkaline phosphatase.) Iodoacetate, however, was found to inhibit both the red cell and the prostatic acid phosphatases nearly to the same extent. This result is against the hypothesis that thiol groups are not necessary for the activity of the prostatic enzyme, unless this inhibition is due to some other reason than combination with thiol groups. On the other hand, the relative ineffectiveness of this reagent as thiol inhibitor has often been pointed out in the literature, and many well known thiol enzymes have been found to be unaffected by iodoacetate (Singer, 1948, Hopkins, Morgan & Lutwak Mann, 1938). The possibility that the thiol groups in the prostatic enzyme are attacked by certain reagents but not by others cannot be overlooked. This point needs more investigation using a variety of effective thiol reagents. The inhibition of sulphhydryl enzymes by heavy metals has been successfully reversed by thiols, e.g. cysteine or glutathione (Hellerman, 1937, Barron & Kalnitsky, 1947). In the present experiments the inhibition of the red cell phosphatase by copper could not be reversed by thiols. It seems that the copper mercaptide combination of the red cell enzyme is so stable, that, unless an appropriate thiol compound is able to dissociate the enzyme-copper complex, the inhibition is irreversible. Neither cysteine nor glutathione seems to have the power to do so.

The importance of the nature of the buffering substance in the study of activators and inhibitors on phosphatases has been pointed out by Lundquist (1947), who found that the hydrolysis of phosphorylcholine and β glycerophosphate by the prostatic enzyme is much faster in citrate buffer than in acetate buffer (10-30 and 100-500% respectively). This may be the reason for the very slow hydrolysis of β glycerophosphate by red cells in acetate buffer, and might furnish a useful clinical method for the determination of prostatic acid phosphatase in serum in methods where β glycerophosphate is used as substrate. The same author also found that citrate buffer prevents the inhibition of the prostatic enzyme by certain other organic acids, e.g. maleic.

The inhibition of acid phosphatases other than those of normal plasma and red cells by L tartrate is particularly interesting since it is stereoisomeric in nature and is highly specific. The application of this phenomenon is of considerable diagnostic value in elucidating the slightly raised values for acid phosphatase in the serum in doubtful cases of prostatic cancer. It has been tried in several cases and

the results were very satisfactory. In this respect, the use of L tartrate is similar to, but is better than, Herbert's (1946) technique of incubation with ethanol. It does not interfere with the corpuscular enzyme, and thus haemolysis causes no error, and the plasma acid phosphatase in non malignant cases is not affected by L tartrate. This was established by trying the effect of L tartrate on plasma acid phosphatase in forty nine different non prostatic cases. In about 80% of these cases the total acid phosphatase was either not affected, or only very slightly affected (± 0.5 unit) by L tartrate, while when the same plasma were subjected to the ethanol technique only 40% of them were unaffected. Even in liver disease, where there is a raised total acid phosphatase in most cases, these high values were not significantly affected by L tartrate.

The liver acid phosphatase was found to be inhibited by L tartrate, while the bile enzyme was not affected (cf Table 11). This may be due to certain substances present in the bile that protect the enzyme against such inhibition. Thus the slightly high serum acid phosphatase which occurs during liver disease and which, in many cases, is accompanied by high concentrations of bile constituents, would be expected not to be affected by L tartrate. This protective action may be due to bile salts which inhibit other acid phosphatases, but have no such effect on the plasma enzyme.

The above facts provide sufficient evidence to conclude that the acid phosphatases of the prostate gland and of the red cell are different enzymes.

Differences amongst the acid phosphatases of other tissues are also demonstrable. For example, the red cell enzyme is not identical with that of the spleen, as was indicated by Davies (1934). The kidney acid phosphatase also does not seem to be identical with the liver or spleen enzymes, since the former is partially inhibited by ethanol, while the two latter enzymes are not affected. Kochakian & Fox (1944) studied the effect of castration and testosterone propionate on the acid and alkaline phosphatases of the kidney, liver and intestine of the mouse. They found that the activity of the acid phosphatase of kidney was increased with testosterone propionate and decreased by castration while the liver and intestinal enzymes were not affected.

Stilboestrol, together with other oestrogens, is widely used for the treatment of prostatic cancer. As a sequence of favourable response to treatment the plasma acid phosphatase values, which are particularly high in patients with secondaries in the bones, fall markedly during the course of oestrogenic therapy. It was not known whether stilboestrol has a direct inhibitory action on the prostatic and other tissue acid phosphatases, but the present investiga-

tion shows that it has not. The following clinical case illustrates the indirect effect of oestrogens on the prostatic acid phosphatase *in vivo*.

A young man 25 years old was admitted to hospital complaining of pain under the sternum for 3 weeks, cough and dyspnoea for 5 days. He was well built and hair distribution and external genitals were normal. Both breasts were visibly enlarged, there was no other significant physical sign. The Aschheim Zondek test was positive with a urine dilution of 1:10, and a serum dilution 1:100, giving a concentration of 4000 to 40,000 and between 40,000 and 400,000 mouse units of gonadotrophic hormone/l. The blood count was significantly abnormal. He was regarded as a case of generalized chorion epithelioma, primary unknown, there being no palpable lesion of the testes. The patient deteriorated rapidly and died. The summary of lesions found on post-mortem examination is as follows: (1) chorion epithelioma arising in superior mediastinum presumably in the teratoma, (2) massive pulmonary metastases, (3) gynecomastia. There was one metastasis, 3 mm diameter in the inferior part of the left lobe of the cerebellum, the brain was otherwise normal.

The two breasts were dissected out and weighed 95 g. Macroscopically they resembled a non lactating female breast. They were sharply defined from the surrounding fat. They were examined for alkaline phosphatase and were found to be very poor in this enzyme.

The thyroid, parathyroid and pituitary were normal. The cortex of the suprarenal appeared somewhat thinned. Both testes were of normal size and quite free from tumour or any abnormality. The seminal vesicles appeared enlarged. The prostate gland appeared quite normal in size. It was examined for phosphatase activity, and it was found that 1 g wet weight of prostate liberated 126 mg phenol from phenyl phosphate at pH 5, during 1 hr at 37°. The normal human prostate gland liberates 3000–10,000 mg phenol/g wet weight under the same conditions.

It is, therefore, believed that the gonadotrophic hormones, through their indirect action on the prostate cells, cause less phosphatase to be produced. A parallel action is believed to take place when oestrogens are administered in the treatment of cancer of the prostate with secondaries.

SUMMARY

1 The prostatic and red cell acid phosphatases have been studied with respect to pH hydrolysis curves of phenyl phosphate, the relative rates of hydrolysis of α and β glycerophosphates, and the effect of magnesium. The presence of two acid phosphatases in the red cells has been confirmed.

2 The effects of calcium, chromium, cobalt, copper, iron, manganese, nickel and zinc on the activity of the prostatic and red cell enzymes are described. No evidence for enzyme activation by any of these metals was obtained. Copper completely destroys the red cell enzyme, while iron, in acetate buffer, strongly inhibits the prostatic enzyme, but has no such action in citrate buffer.

3 Glycine and alanine have no effect on either prostatic or red cell phosphatases. Cysteine slightly activates both enzymes.

4 The effects of ethanol, acetone, formaldehyde and certain organic acid radicals on acid phosphatase activity have been studied. L Tartrate,

while not affecting the acid phosphatases of plasma and red cells, inhibits other acid phosphatases particularly that of the prostate, and the nature of this action has been studied and discussed.

5 Stilboestrol has no direct effect on any of the acid phosphatases.

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The Micro- and Ultramicro-determination of Chlorine in Organic Compounds

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(Received 9 July 1948)

To follow the metabolism of 1 1 1 trichloro 2 2 di-(*p* chlorophenyl) ethane (DDT) in tissues a method was necessary for determining small amounts of organically bound chlorine. In addition, a technique was required for the estimation of DDT in the presence of possible degradation products. Although the determination of inorganic chloride in minute amounts is possible (Sendroy, 1937, 1939, Conway, 1935), adaptation to the new requirements would not be convenient since in Sendroy's method chloride is precipitated with silver iodate and the concentration of chloride must be at least 0.01 M. The method of Conway is not subject to these limitations, but the dimensions of his microdiffusion unit are such that it would be difficult to make estimations of small amounts of organically bound chlorine in relatively large amounts of tissue.

The basis of the present method is the photometric determination of the intensity of the starch-iodine blue colour after liberation of free iodine from potassium iodide by chlorine formed by the oxidation of the compound under test.

A technique has also been devised for the determination of DDT in tissue extracts in the presence of metabolites. The discovery by White & Sweeney (1945) that 2 2 di-(*p* chlorophenyl)acetic acid (DDA) is a product of DDT metabolism in animals has made it possible to separate an alkali soluble from a neutral fraction of organically bound chlorine found in the tissues of animals after the ingestion of DDT.

METHODS AND RESULTS

Oxidation of organically bound chlorine is effected by heating with specially prepared silver dichromate in the presence of concentrated sulphuric acid. Inorganic chloride may be determined after its oxidation in the cold by an acid permanganate mixture (Conway, 1935), organically bound chlorine not being attacked under these conditions. The latter method may be used for the specific determination of DDT since 1 mol of hydrochloric acid is liberated/mol of DDT by the action of alcoholic alkali (Gunther, 1945). Chlorine volatilized by the oxidation is carried

by a stream of nitrogen into potassium iodide solution contained in an absorbing vessel. The problem then becomes one of micro iodometry. For amounts down to $1\mu\text{g}$ chlorine a direct determination of the liberated iodine is possible. For amounts below $1\mu\text{g}$ a technique has been devised for the separation of the free iodine and its conversion to iodate.

In studies of the distribution and fate of DDT in the body, these methods may be applied to the analysis of tissue extracts. By carrying out determinations of total chlorine, inorganic chlorine before and after incubation with alcoholic alkali and of alkali soluble organically bound chlorine, it is possible to establish the presence of DDT, other organic compounds containing neutral chlorine and alkali soluble compounds containing chlorine.

Apparatus

Description The apparatus (Fig. 1) is constructed of pyrex glass. Oxidation is carried out in the reaction vessel *A*, connected by means of combined still head and dropping funnel *B* to the absorbing vessel *C* which consists of a delivery tube contained in an outer vessel, the walls of which fit closely to the spiral rod wound round the delivery tube. *C* is connected with the absorber *D* of similar construction. The central absorber tube *C* is required only for the determination of Cl in tissues. Its purpose is to remove SO_2 which may be evolved during the reaction and it contains a mixture of saturated KMnO_4 (2 vol) and 50% (v/v) H_2SO_4 (1 vol).

A spiral of glass rod is wound round the stem of the dropping funnel *B*, its purpose is made plain in the section on the determination of DDT. The gas inlet *E* is connected by pressure tubing to a N cylinder fitted with a reducing valve, and the gas rate is adjusted by the bubble rate in a wash bottle. The gas inlet consists of a tube fitting by a standard joint into the neck of the dropping funnel *B*. The latter holds H_2SO_4 , which is run into the flask after the apparatus has been assembled.

The purpose of a fine capillary in *C* and *D* just below the level of the joint socket will be made clear in the section on gas flow.

Gas flow Because of the dimensions of the apparatus, the passage of gas at the rates required leads to irregular flow owing to fluctuations of pressure within the assembly. Regularity was achieved by introducing constrictions just below the joints of the delivery tubes (Fig. 1).

Reagents

Sulphuric acid The concentrated acid (A.R. grade) has been found to give a zero blank. It should be stored away from direct light and should be shaken before use with a small amount of silver dichromate to remove reducing substances.

Silver dichromate. The commercial salt yields a large blank, owing to traces of nitrate. The following method of preparation yields a product giving zero blank values. Silver oxide is prepared from AgNO_3 by precipitation with 40% (w/v) NaOH . The precipitate is washed with water 12 times by decantation. The moist oxide is then dissolved in the minimum volume of 70% (v/v) H_2SO_4 and an excess of CrO_3 , dissolved in the minimum volume of water, is added. A dark

red precipitate of the dichromate separates at once. The flask is now placed in a boiling water bath, and air drawn rapidly through the mixture for about 3 hr. Nitrate and chloride are thus completely removed. As soda-lime tubes should be attached to the air intake. The contents of the flask are then diluted and allowed to stand overnight. The precipitate is washed by decantation at least 10 times. At the end of the washing, the water should be bright yellow in colour from dissolved dichromate. The flask and contents are now dried at 90° and the silver dichromate stored in a dark bottle. The yield is almost quantitative.

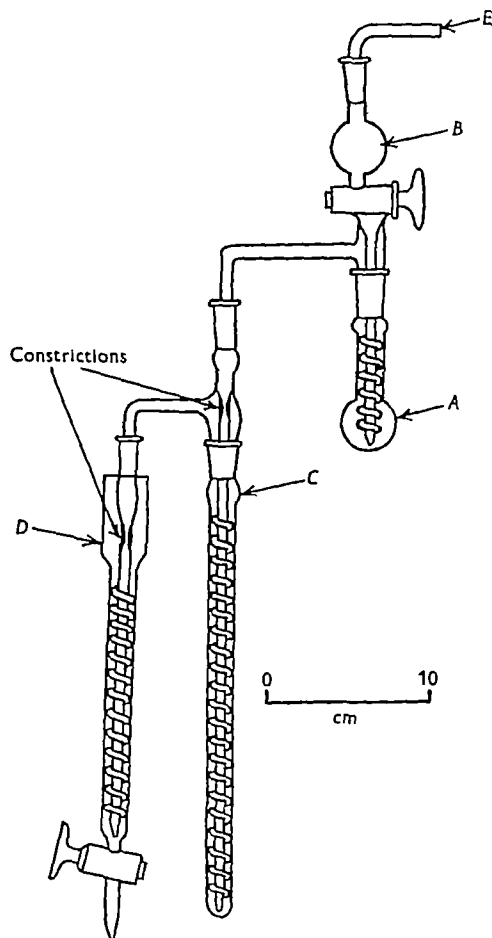


Fig. 1 Apparatus for liberation of chlorine and absorption train.

Potassium iodide A 5% (w/v) KI (A.R.) solution is used as the absorbing fluid. It should be freshly made daily in water redistilled over Ba(OH)_2 in a glass apparatus.

Starch A 1% (w/v) solution of starch (A.R.) is prepared by suspending 1 g starch in 10 ml cold glass distilled water. About 90 ml of water is brought to the boil and the starch suspension added rapidly to it. The solution is allowed to boil for 1 min. and then made up to 100 ml.

n Butanol Commercially obtainable pure *n* butanol is suitable. It is stored in a cold room to avoid formation of aldehyde, but discarded after 1 month.

Light petroleum The A R grade, boiling range 40–60°, must be purified by washing 6 times with conc H_2SO_4 and 6 times with water. It is then dried and redistilled.

Reagents for the Groat (1934) oxidation These are listed by Salter (1937)

Solvents Acetone (A R) was redistilled over anhydrous K_2CO_3 , ether (A R) was redistilled over Na.

Methanolic KOH is prepared from methanol redistilled over Na, KOH (A R) gives a small blank value. Each fresh batch of methanolic KOH should be standardized with respect to chlorine content.

Potassium permanganate (A R) is recrystallized until it yields no blank.

Microestimation (1.0–120 μg chlorine)

Method for pure substances The compound, in a suitable volatile solvent, is introduced into the oxidation flask (Fig 1, A) and the solvent is evaporated carefully under reduced pressure so that no traces are left. Failure to observe this precaution will lead to violent reactions when oxidation starts and may give erroneous results. Silver dichromate (0.5–1.0 g) is introduced and the apparatus is assembled without tube C. The absorber D is filled with KI solution (5 ml) and water up to the shoulder of the vessel. Conc H_2SO_4 (8 ml) is run into the dropping funnel B, the gas flow started at 10 ml/min, and the tap of the dropping funnel turned so that the acid is forced into the flask. The flask is now heated to 110° in a glycerol bath. Gas flow is continued for 30 min after the correct bath temperature is reached. The liberated I_2 is then estimated. For amounts of 1–100 μg Cl a direct reading is easily possible using the starch iodine blue colour and an absorptiometer. For amounts over 100 μg titration with 0.005N $\text{Na}_2\text{S}_2\text{O}_3$ is recommended.

Estimation of the starch iodine blue colour The contents of the absorber are transferred completely to a volumetric flask which is filled nearly to the mark with water and 0.5 ml of 1% (w/v) starch added. The volume is made up, the contents of the flask well mixed, and any blue colour which develops read within 10 min. at 25°. Using a single cell photoelectric absorptiometer with 2 cm cells and an Ilford red filter (no 204), it was found that a 25 ml. final volume gave a readable blue colour within the limits 1–25 μg Cl, with a final volume of 50 ml the upper limit is 50 μg and a final volume of 100 ml raises this to 100 μg .

Effect of iodide concentration Bairstow, Francis & Wyatt (1947) have shown that in the presence of a large excess of KI (or indeed of any salt) starch will give a purple red colour with iodine. Conway (1935) has used this colour in his micro determination of Cl, but with the absorptiometer used in these experiments the readings obtained were found to deviate from Beer's law. The bearing of this point on the present method is that with a final volume of less than 25 ml and in the presence of 5 ml of 5% (w/v) KI, the starch iodine complex gives a purple red colour, with the dilutions and quantity of KI recommended in the present paper a satisfactory blue colour is obtained.

Effect of temperature In accordance with the findings of other workers (Bairstow *et al* 1947, Sendroy, 1937), the starch iodine blue colour has been found to vary in intensity with temperature. A working temperature of 25° gives reliable results.

Addition of starch Starch should be added just before the volume of the solution is finally adjusted, otherwise a purple

red colour may be obtained instead of the usual blue, further dilution does not necessarily result in the conversion of the former to the latter colour.

Time of reading In contrast to other work (Bairstow *et al* 1947, Talbot, Butler, Saltzman & Rodriguez, 1944) it has been found that the blue colour is fairly stable in neutral solution and hence the time of reading is immaterial within wide limits. Fig 2 illustrates this point. Curve A was obtained from repeated estimations of the starch iodine blue colour corresponding to 10 μg chlorine. In contrast, curve B was obtained by the action of acid iodate on KI and indicates a steady deepening of the starch iodine blue colour with time as was expected, since KI decomposes in acid solution.

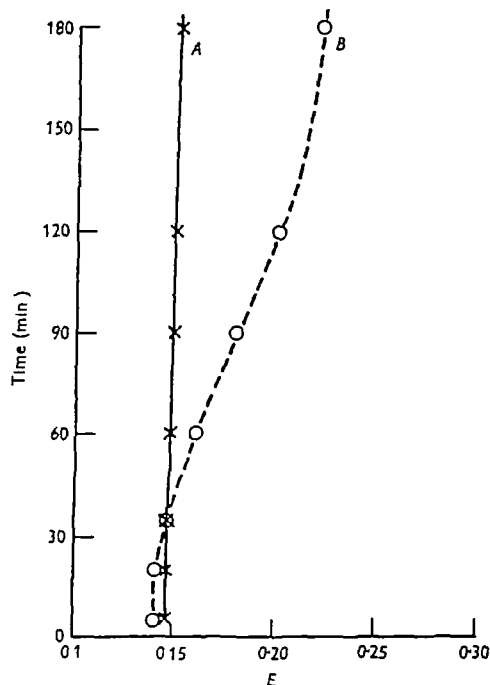


Fig 2 Effect of time on starch iodine blue colour. Iodine equivalent to 10 μg chloride A, starch iodine blue colour in neutral solution, B, starch iodine blue colour obtained by action of acid KIO_3 on KI.

Calibration curves These should be prepared separately for each final volume by carrying out a series of determinations on a standard substance and checked by parallel readings on a standardized iodine solution. The calibration curves obtained by the present method have all been straight lines passing through the origin.

From the results of the analysis of two chlorine containing organic compounds it will be seen that satisfactory accuracy is obtainable (Table 1).

Ultramicro method

For the ultramicro procedure, certain changes are necessary. While it is possible to use the above described absorber (Fig 1, D) with good results one of the same pattern, but of half the volume, is more convenient.

Table 1 *Determination of the chlorine content of two organic substances by the micro method*

Amount (μg)	Calc (μg)	Cl content			No of determina- tions
		Found			
		Mean (μg)	s d (μg)	Range (μg)	
DDT					
120	60.0	60.25	1.89	57-61	4
100	50.0	50.10	1.57	48-52	6
75	37.5	38.30	1.00	37-39	3
50	25.0	24.80	0.80	24-26	5
25	12.5	12.75	0.27	12-13.5	6
10	5.0	4.80	0.35	4.5-5.3	4
5	2.5	2.63	0.29	2.3-3.0	4
<i>p</i> Chlorobenzoic acid					
499.0	113.0	114.25	1.89	113-117	4
249.5	56.5	56.75	1.26	55-58	4
99.8	22.60	22.90	1.72	21-24	3
49.9	11.30	11.13	0.713	10.3-11.6	3

In this method the iodine is extracted from potassium iodide solution by an immiscible solvent, the solvent is then washed free from iodide and the iodine converted to iodate. The starch iodine blue colour is used for its determination. In a final volume of 25 ml the intensity of the blue colour allows the estimation of chloride as low as 0.25 μg . With the Rehberg or similar microburette, a titrimetric method can be used.

The technique up to the extraction of the iodine is similar to that already described for the micro procedure. Silver dichromate (250-500 mg) with 5-8 ml H_2SO_4 is used as the oxidizing mixture. After the passage of the gas for 30 min, the fluid in the absorber is rinsed into a small separating funnel with two washes of water. Extraction is accomplished with a mixture of light petroleum (45 vol) and *n*-butanol (55 vol), vigorous shaking should not be used. The butanol-petroleum layer is transferred to a second separating funnel, and the aqueous layer extracted once more with solvent mixture and the extracts combined. The latter are generally opaque and may be cleared by washing with saturated Na_2SO_4 solution. Seven further washings are carried out, water and Na_2SO_4 solution being used alternately with vigorous shaking. I_2 is next converted to iodide by adding to the separating funnel 0.5 ml M K_2CO_3 and 0.5 ml 0.1 M sodium metabisulphite. The two layers are mixed and allowed to separate. The aqueous layer is run into a small vessel, to which is added two further washes of 1 ml portions of water. Increase in the volume of aqueous washes to more than 3 ml, and violent shaking should be avoided since they increase the amount of the organic solvents in the aqueous layer and these may interfere with the subsequent oxidation of iodide to iodate according to Groak (1934).

The iodide containing solution is now heated on a boiling water bath for 30 min, after which there should be little of the organic solvents present. The vessel is now transferred to a water bath at 80°, and 15-20 drops of N KMnO_4 are carefully introduced directly into the liquid. The solution should remain pink. The vessel is kept at 80° for 4 min, and then 6 drops of 8 N H_2SO_4 are added down the side of the vessel and the latter replaced in the water bath for 4 min. The solution should remain pink to the end of this step.

NaNO_2 (0.75 N) is now added dropwise with shaking until the solution is colourless. The sides of the vessel are rapidly washed down with a stream of water, followed by 3 drops of 5 M urea. The vessel is again heated on a boiling water bath for 30 min and then put in ice. When cold, the solution is transferred to a 25 ml volumetric flask and 0.5 ml of 1% (w/v) starch solution added, followed by water nearly up to volume, 0.5 ml 1% (v/v) KI and water to 25 ml. The intensity of any blue colour developed is read within 5 min at 25°. Since the colour is developed in an acid medium, the time of reading is critical.

In carrying out this series of steps it is essential to adhere rigidly to the technique. Where the extraction of I_2 is concerned, for example, it is a matter of convenience what volumes of the petroleum-butanol mixture are used, except that washing free of iodide is facilitated by the use of small volumes, on the other hand, the removal of the I_2 from the organic solvents must be carried out in the minimum of the aqueous solution.

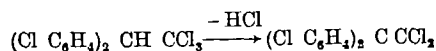
The Groak (1934) reaction must be carried out with the utmost care, the solutions being added according to direction, only the 8 N H_2SO_4 should be run down the side of the vessel, all the other reagents being added directly to the solution. It must be stressed that the solution should remain pink during the first 8 min of the process; this should be the case if the organic solvents have been removed. The analytical results given in Table 2 illustrate the degree of accuracy obtainable.

Table 2 *Determination of chlorine content of DDT by the ultramicro method*

Amount of DDT (μg)	Calc (μg)	Cl content			No of determina- tions
		Found			
		Mean (μg)	s D (μg)	Range (μg)	
2.00	1.0	0.92	0.265	0.8-1.3	5
1.50	0.75	0.75	0.234	0.6-0.85	5
1.00	0.50	0.525	0.32	0.4-0.6	4
0.50	0.25	0.25	0.186	0.2-0.35	3

The determination of DDT

Gunther (1945) showed that when DDT is incubated with alcoholic potassium hydroxide, the following reaction takes place



Wain & Martin (1947) showed that this reaction takes place at room temperature. Following Conway (1935), the inorganic chloride so liberated was oxidized in the cold with acid permanganate, the organic by-product of the reaction and DDT itself are not attacked by the cold mixture. Volatilization of chlorine from the reaction flask depends upon thorough agitation of the acid permanganate mixture by the gas stream. A spiral (Fig. 1, A), wound round the stem of the dropping funnel, ensures this and increases the effective surface. The flask is filled with the oxidizing mixture so that the fluid level is well up the stem.

The technique which follows is also applicable to the determination of inorganic chloride

Procedure A solution of DDT is placed in the oxidation flask and the solvent evaporated. Methanolic KOH (0.1N) is added and the mixture maintained at 30° for 0.5 hr. A little water is then added and the methanol removed under reduced pressure. Where amounts below 25 µg DDT are analysed, 0.02N KOH may be used. When evaporation is complete, 12–15 ml of a mixture of saturated KMnO_4 (1 vol) and 25% (v/v) H_2SO_4 (1 vol) is run in, and the determination carried out as for organically bound Cl but without heating.

The analyses recorded in Table 3 illustrate the applicability of the method.

Table 3 *Determination of alkali labile chlorine of DDT and of sodium chloride*

Amount (μg)	Calc (μg)	Cl content			No of determina- tions
		Found			
		Mean (μg)	s d (μg)	Range (μg)	
DDT					
100.0	10.0	9.60	0.314	9.0–11.5	6
50.0	5.0	4.97	0.182	4.0–6.3	6
10.0	1.0	0.97	0.22	0.8–1.3	6
5.0	0.5	0.51	0.31	0.4–0.65	6
2.5	0.25	0.24	0.19	0.2–0.35	4
NaCl					
116.92	70.92	69.6	1.7	67.0–72.0	6
58.46	35.46	35.6	1.29	34.0–37.0	6
29.23	17.73	17.7	0.53	17.0–18.7	6
11.69	7.09	6.9	0.118	6.5–7.5	6
5.846	3.546	3.6	0.120	3.0–4.0	6
1.169	0.709	0.70	0.103	0.60–0.85	6

Estimation of DDT and allied substances in tissues

The methods described above can be applied to extracts made from tissues. Certain modifications of the procedure are required. The tissue extracts usually contain a rather large amount of fatty and other substances which react violently with the oxidizing mixture to liberate sulphur dioxide. This interferes with the liberation of iodine in the absorber. To obviate this risk, a central absorber (Fig. 1, C) is placed between the oxidation flask and the absorption vessel containing potassium iodide solution. It contains about 10–15 ml of a mixture of saturated potassium permanganate solution (2 vol) and 50% (v/v) sulphuric acid (1 vol). This absorbs all the reducing agents volatilized, but not the chlorine.

Extractions of tissues Tissues (1–2 g wet tissue, or as much as is available) are ground with anhydrous Na_2SO_4 (A.R.) until a freely flowing dry powder is produced. During the grinding, enough 8N H_2SO_4 is added to bring the pH to 2. The powder is transferred completely to a flask and extracted by three successive 35 ml portions of boiling ether. Each portion of ether is allowed to cool and filtered into a second flask through a filter paper (Whatman no. 1).

The combined filtrates are transferred to a separating

funnel and shaken with 10 ml 10% (w/v) NaOH. The aqueous layer is run into a second separating funnel. The ethereal layer is washed with 10 ml water which is added to the alkaline wash. The combined aqueous washes are extracted with 10 ml ether and the ethereal extract added to the first separating funnel. The combined ethereal extracts are washed twice with water and evaporated, the residue taken up in acetone, filtered if necessary and adjusted to a convenient volume (10–20 ml). This solution contains all the neutral organic Cl. Portions are used to determine total organic Cl and DDT (as alkali labile Cl). A determination of entrained inorganic Cl, i.e. Cl fortuitously extracted from the tissue, is also carried out and the values for the total and alkali labile Cl are corrected by this amount. The combined alkaline extract, containing alkali soluble organic Cl, is acidified to pH 2 with 8N H_2SO_4 and extracted by shaking vigorously with an equal volume of ether. The ethereal solution is evaporated, the residue taken up in acetone, which is filtered if necessary and portions of the filtrate analysed for total and inorganic Cl, the value for alkali soluble organic Cl being obtained by deduction.

Table 4 *Recovery of DDT and di(p chlorophenyl) acetic acid (DDA) added to wet tissues*

(Wt of wet tissue 2 g in each experiment)

Tissue	Wt of substance added to tissue		Wt found	
	DDT (µg)	DDA (µg)	DDT (µg)	DDA (µg)
Kidney	25.0	50.0	25.0	52.0
	10.0	25.0	11.0	23.5
	5.0	0.0	5.0	0.0
Liver	10.0	5.0	11.0	4.5
	25.0	100.0	27.0	103.0
	40.0	55.0	36.0	52.0
Muscle	0.0	25.0	0.0	22.0
	50.0	60.0	54.0	57.0
	20.0	10.0	20.0	8.0
Brain	20.0	10.0	20.0	8.0

Table 4 illustrates the recovery from tissues of added DDT and DDA. It will be seen that satisfactory results can be obtained.

DISCUSSION

The apparatus described resembles that of Kramick & Zacherl (1937) but the dimensions differ, and the introduction of the intermediate absorber makes it possible to apply the method to the analysis of tissue extracts. The differences in the mode of determination may be put shortly by saying that the Kramick & Zacherl method is intended to determine relatively large amounts of chlorine in a small amount of organic material, while the present method is designed for the determination of small amounts of chlorine in relatively large amounts of organic material.

The accurate application of the present method to tissue determinations depends on the securing of tissue extracts which are relatively free from inorganic chloride. However, a blank is always

obtained and parallel determinations of inorganic chloride must be carried out. Using the micro method, a direct determination of $1.0\mu\text{g}$ chlorine is possible. The ultramicro method is intended for use with amounts of tissue providing less than $1.0\mu\text{g}$ of chlorine, e.g. tissue slices. It is hoped that, by reducing the size of the apparatus and by modifying the determination of iodine, amounts of chlorine down to $0.1\mu\text{g}$ may be determined. Such a technique might be useful for investigations on the metabolism of DDT in insects.

It should be pointed out here that the application of the method to substances other than those specifically mentioned may be limited by their volatility. For example, consistently low results were obtained when estimations were attempted on chloroacetamide. This was attributable to volatilization of the substance before complete oxidation could take place.

SUMMARY

1. Methods are described for the micro and ultramicro determination of chlorine in DDT and related compounds, applicable to amounts of $1\text{--}120\mu\text{g}$ and $0.25\text{--}1.0\mu\text{g}$ of chlorine, respectively.

2. The techniques have been successfully applied to the analysis of tissues containing these substances.

My thanks are due to Prof. G. R. Cameron, F.R.S. for his kindness and interest in this work and the experiments still in progress, to Prof. C. Rimington for his advice and criticism, to Dr D. R. Willkie, University College, London, Messrs J. S. Pizey and J. C. Godding, and finally to Mr J. Trendall, glassblower, University College, London, without whose skill and ingenuity this work would have been made immeasurably more difficult.

The writer is indebted to the Medical Research Council for a maintenance grant, and to the Agricultural Research Council and Graham Research Fund of the University of London for expenses grants.

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Studies in Detoxication

25 THE CHARACTERIZATION OF PHENYLGLUCURONIDE, AND ITS RATE OF HYDROLYSIS COMPARED WITH THAT OF PHENYLSULPHURIC ACID

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(Received 14 December 1948)

The present work was carried out for two reasons. First, investigations in this laboratory on the metabolism of benzene showed that 11% of orally administered benzene was excreted by rabbits as glucuronides (Porteous & Williams, 1949a), and Schmiedeberg (1881) isolated from the urine of a benzene-fed dog a crystalline substance which was probably phenylglucuronide. We therefore required phenylglucuronide as a reference compound for further investigations on benzene. Secondly, phenylglucuronide is used as a standard substrate for the

assay of β -glucuronidase in tissues (see Kerr, Graham & Levy, 1948; Mills, 1948). After a search of the literature we felt that phenylglucuronide had not been properly described.

Phenyl β -D-glucuronide was first isolated by Kulz (1890) from the urine of rabbits receiving phenol by injection. His material melted at 148° , but he attributed the wrong formula, $\text{C}_{12}\text{H}_{16}\text{O}_7$, in accordance with his analytical figures. It was synthesized from phenol and acetobromoglucurone by Neuberg & Niemann (1905), who described it as anhydrous, m.p. $150\text{--}151^\circ$, $[\alpha]_D^{17} - 83.3^\circ$ (solvent not mentioned). For material isolated from the urine of a sheep fed with phenol, Salkowski & Neuberg (1906) gave m.p. $148\text{--}150^\circ$, $[\alpha]_D - 81.9^\circ$ (solvent not mentioned) and analytical figures for the

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anhydrous glucuronide ($C_{12}H_{14}O_7$). Masamune (1933), who isolated the compound from rabbit urine after phenol feeding, dried it *in vacuo* at 78° and his analytical figures agreed with those for anhydrous phenylglucuronide. He gave *m p* 160° (decomp), but quoted no specific optical rotation.

It is clear, therefore, that phenylglucuronide has not been satisfactorily described. We shall show that phenylglucuronide is normally a dihydrate unless dried at elevated temperatures. Its rate of hydrolysis by acid compared with that of phenylsulphuric acid has also been studied with a view to justifying a procedure used in this laboratory for separating phenols conjugated with sulphuric acid from those conjugated with glucuronic acid.

EXPERIMENTAL AND RESULTS

Phenylglucuronide

(a) *Isolation from urine* The isolation of the glucuronide as the basic salt has been described by Porteous & Williams (1949a). Phenol was fed at a dose level of 0.8 g to rabbits (3 kg), and from an 18 hr urine the yield of crystalline glucuronide was 0.9–1.0 g/g of phenol fed. To purify the glucuronide it was dissolved in absolute ethanol and the solution was filtered and evaporated to dryness *in vacuo*, the residue was recrystallized from the minimum of hot water. After drying *in vacuo* at room temperature the dihydrate of phenylglucuronide was obtained as long colourless needles, *m p* 161 – 162° (corr, decomp) after sintering at 110 – 115° . It showed $[\alpha]_D^{27} - 78.5^\circ$ (c, 2 in water) (Found C, 47.5, H, 5.9, H_2O , 11.7, C_6H_5OH , 30.0%, equiv by titration, 299. $C_{12}H_{14}O_7 \cdot 2H_2O$ requires C, 47.1, H, 5.9, H_2O , 11.8, C_6H_5OH , 30.7%, equiv 306). After recrystallization from benzene ethanol the compound retained its water. It is non-reducing and gives a strong Tollens naphthoresorcinol reaction.

On drying to constant weight at 105° (1.5–2 hr) anhydrous phenylglucuronide as described by Masamune (1933) was obtained. This formed white needles, *m p* 161 – 162° (corr, decomp) and $[\alpha]_D^{18} - 90.5^\circ$ (c, 1.6 in water) (Found equiv 272. Calc for $C_{12}H_{14}O_7$, equiv 270). The anhydrous compound dissolved less rapidly in water than the dihydrate.

It is probable that the optical rotations quoted by Neuberg & Niemann (1905) and Salkowski & Neuberg (1906) are for the dihydrate and that their analytical figures are for dried samples.

(b) *Benzylamine salt of phenylglucuronide* The dihydrate (200 mg) was dissolved in 20 ml. ethyl acetate containing 5% (v/v) absolute ethanol. Benzylamine (10 drops) was added until no further precipitation occurred. The benzylamine salt (200 mg) was recrystallized from 95% ethanol and formed colourless needles, *m p* 207 – 208° (corr, decomp), $[\alpha]_D^{25} - 62.3^\circ$ (c, 2 in water) (Found N, 3.5%, $C_{13}H_{17}O_7N$ requires N, 3.7%). It is easily soluble in water, insoluble in cold but soluble in hot ethanol. It gives the Tollens reaction readily.

(c) *Absorption spectra* The ultraviolet absorption spectra (determined with a Hilger E3 Quartz Spectrograph) of phenylglucuronide and its dihydrate were identical. The common curve is shown in Fig 1. From the values of $E_{1\%}^{1\text{cm}}$ for the anhydrous and hydrated forms at various wave-

lengths, the molecular weight of the hydrate could be calculated from that of the anhydrous form (mol wt 270). For the wavelengths 271, 268 and 259.5 $m\mu$ the values 305, 311 and 309 respectively, were found (calc mol wt 306), thus confirming that the compound was a dihydrate.

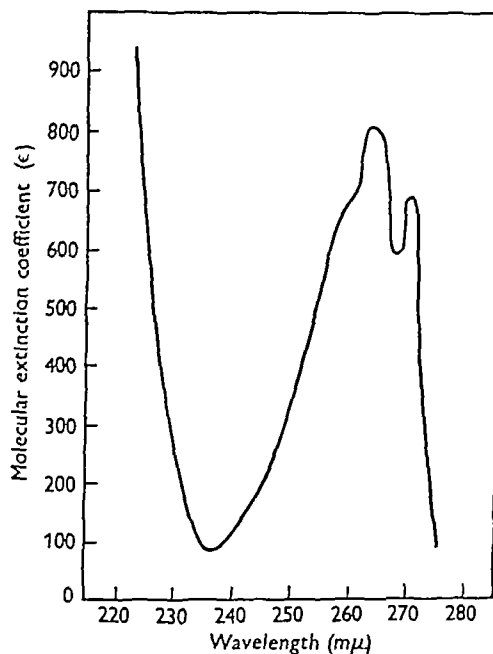


Fig 1 Ultraviolet absorption spectrum of phenylglucuronide or its dihydrate in water. λ_{\max} 271 and 264.5 $m\mu$. (ϵ_{\max} 690 and about 810, respectively), and $\sim 259.5 m\mu$. (ϵ_{\max} 710).

The spectra (Fig 1) show bands with λ_{\max} 271 and 264.5 $m\mu$ (ϵ_{\max} 690 and about 810, respectively), and an inflexion at 259.5 $m\mu$ with ϵ_{\max} 710. It is interesting to compare these values with those for phenol in ionizing and non-ionizing media. Phenylglucuronide (C_6H_5OR , where $R = C_6H_4O_6$) should be similar to an ionized phenol ($R = H$). Phenol in pentane (non-ionizing medium) shows maxima at λ_{\max} 270 and 277.5 $m\mu$ (ϵ_{\max} 2000 in both cases) and a small band at 265 $m\mu$ (ϵ_{\max} 1270, Klingstedt, 1923). In ionizing solvents phenol shows a single large band in this region of the ultraviolet, thus in water this band has λ_{\max} 269.8 $m\mu$ (Klingstedt, 1922) and in ethanol λ_{\max} 273 $m\mu$ (Morton & Stubbs, 1940). The spectrum of phenol in various solvents is further discussed by Stimson & Reuter (1945).

The rate of hydrolysis of phenylglucuronide by acid compared with that of phenylsulphuric acid

It has been observed on several occasions in this laboratory that glucuronides of phenols are not rapidly hydrolysed by dilute acids at the temperature of a boiling water bath, whereas ethereal sulphates of phenols are readily hydrolysed. We have made use of this observation for the separation of phenols conjugated with sulphuric acid from those

conjugated with glucuronic acid (Garton & Williams, 1948, 1949, Porteous & Williams, 1949b, Smith & Williams, 1949). The present experiments were carried out to see how far this procedure was justified and could be made more discriminating

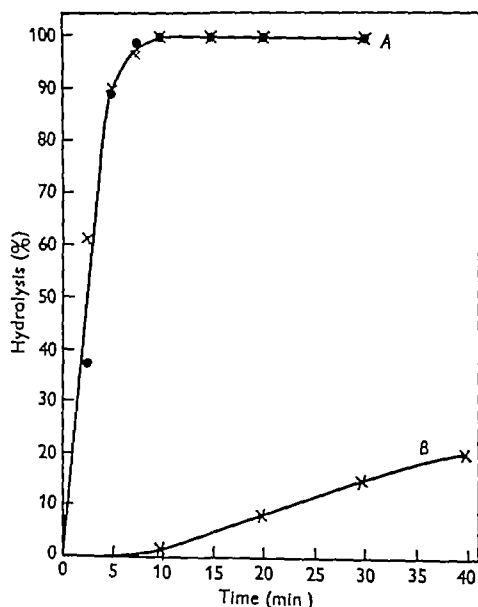


Fig 2 Comparison of the rate of hydrolysis by N HCl of phenylsulphuric acid with that of phenylglucuronide at 93–95° A, potassium phenylsulphate, \times 0.0048M, \bullet 0.0017M B, phenylglucuronide dihydrate, 0.0005M

The potassium phenylsulphate used was prepared according to Burkhardt & Lapworth (1926) (Found S, 15.4% Calc for $C_6H_5O_3SK$, S, 15.1%)

Standard solutions of phenylglucuronide dihydrate (0.0005M) and potassium phenylsulphate (0.0017 and 0.0048M), made N with respect to HCl, were heated in a boiling water bath (temperature of solutions 93–95°). All

solutions were raised to 93–95° before mixing. Samples of 5 ml were withdrawn periodically and immediately neutralized with solid $NaHCO_3$. The mixture was diluted to a known volume with distilled water and its free phenol content determined colorimetrically with 2,6-dichloroquinonechloroimide as described by Porteous & Williams (1949a). The results (Fig 2) show that, at this temperature, phenylsulphuric acid is completely hydrolysed in 10 min whereas the proportion of phenylglucuronide hydrolysed is less than 2%. In previous papers we had used periods of 20–30 min and, although less than 10% of the glucuronide is hydrolysed in this time, it appears that a 15 min hydrolysis under our conditions would have given a sharper separation. In experiments in which the liberated sulphate was estimated, Sperber (1948) showed that phenylsulphuric acid was completely hydrolysed by approx 0.25N HCl in 10 min and resorcinylsulphuric acid in 15 min. Masamune (1933) studied the hydrolysis of phenylglucuronide by N HCl at 100° (i.e. in boiling solution) and found 16% hydrolysis in 10 min and 99% in 3.5 hr. Porteous & Williams (1949a) found that 10N H_2SO_4 was necessary to hydrolyse phenyl glucuronide completely in 1 hr at 100°.

SUMMARY

1 Phenylglucuronide has been prepared biologically and characterized. It normally occurs as a dihydrate, its benzylamine salt has been described.

2 The ultraviolet absorption spectrum of the glucuronide in water has been determined and discussed in relation to that of phenol.

3 Phenylsulphuric acid is hydrolysed by acid at 93–95° at least 50 times as rapidly as is phenyl glucuronide.

We wish to thank Prof R. A. Morton for assistance with determination of spectroscopic data. The expenses of this work were in part defrayed by a grant from the Medical Research Council and one of us (D.R.) participated while holding a Medical Research Council Studentship for training in research methods.

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Some Effects of Glucose and Calcium upon the Metabolism of Kidney Slices from Adult and Newborn Rats

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(Received 23 December 1948)

Cutting & McCance (1946*a, b*) compared the metabolism of slices cut from the kidneys of newborn and adult animals. They found that the respiratory rate of kidney tissue from newborn rats changed little over a period of 2 hr, whilst that of the adult tissue fell off rapidly. Such a decline has often been reported, and Laser (1942) accepted it as a peculiarity of the kidney. Cutting & McCance noted, however, that their slices of adult tissue were visibly disintegrating after 2 hr, and had often lost 40–50% of their nitrogen. These slices were immersed in a solution buffered with phosphate at pH 7.4, similar to that described by Krebs (1933). It contained no calcium, and had about eight times the concentration of phosphate found in mammalian extracellular fluids. Cutting & McCance (1947) also studied the effect of calcium upon the respiration and the loss of nitrogen of kidney slices from adult and newborn rats. Since they believed that calcium would not remain in a solution containing phosphate at pH 7.4, they made their comparisons in a saline solution buffered with phosphate at pH 6.8 and in bicarbonate Ringer at pH 7.4. The adult tissues lost less nitrogen in the presence of calcium, and their respiratory rate was better maintained, but was still far from constant.

The present work was undertaken to extend these observations, and a medium has been devised in which kidney slices from adult rats respired at a constant rate for 4 hr. This technical improvement has enabled the metabolism of slices from adult and newborn rats to be compared in a more reliable fashion, and in general the previous results have been fully confirmed. The new medium is an artificial extracellular fluid containing glucose, Ca^{++} and phosphate in physiological amounts at pH 7.4. It contains, however, no added bicarbonate. Its buffering capacity is less than that of the solutions which have generally been employed, but it gave satisfactory control of pH because kidney produces less acid than do the tissues upon which much of the early manometric work was done. Furthermore, it was hoped by using a smaller concentration of phosphate buffer to mitigate the difficulties which arise from the low solubility of the phosphates of calcium. Some relevant physicochemical data are briefly discussed in an appendix.

EXPERIMENTAL

Preparation of media

A saline solution was prepared by mixing 0.154M NaCl (232 ml), 0.154M KCl (8 ml), 0.154M MgSO_4 (2 ml.) and 0.110M CaCl_2 (6 ml). The mixture was brought cautiously to pH 7.4 with 0.1N NaOH. 12 ml of M/15 phosphate buffer (pH 7.4), prepared by mixing solutions of Na_2HPO_4 and KH_2PO_4 as described by Hawk, Oser & Summerson (1947, p. 636), were then added, pH was controlled both by capillators and by the glass electrode. To avoid precipitation the Ca and phosphate were not brought together in less than the final volume, but the completed solution was stable.

As extracellular fluids contain about 100 mg/100 ml of glucose, this seemed a natural addition to make to the saline. Accordingly, 0.4 ml of 5% glucose was added to each 19.6 ml of the buffered saline immediately before use. The resulting medium contained approximately, in mmol/l., Na^+ , 140, K^+ , 5, Ca^{++} , 2.5, Mg^{++} , 1, Cl^- , 144, P, 3 and SO_4^{--} , 1, giving a total ionic strength of 0.158. It will be referred to as 'medium A 1'. This solution contained the equivalent of the total Ca of serum, but it was anticipated that some Ca^{++} would be fixed by protein from the slices. In some experiments a similar solution (A 2) was used which contained twice as much phosphate buffer and one half as much 0.11M CaCl_2 , giving the same ionic product, but twice the buffering capacity. No appreciable difference was found between the results of experiments in which A 1 and A 2 were used, and these results have been grouped together under the general heading 'medium A'. Several incomplete forms of this new medium were also employed in comparative studies. Medium B was the same as A 1, except that the CaCl_2 was omitted. Medium C was medium A 1 with the glucose omitted. Medium D was the same as C except that the CaCl_2 was also omitted. It gave results similar to those obtained by Cutting & McCance (1946*a*) with their phosphate solution. Since it was found that increasing the concentration of phosphate up to 18 mM made no essential difference in the absence of Ca, the results of a few experiments using the original solution prepared by Cutting & McCance have been grouped among those under the heading 'medium D'.

In a footnote seen when this work was almost completed, Hawk *et al.* (1947, p. 301) recommended a similar solution without the Mg. A few experiments since then have suggested that respiration is better maintained when Mg is included, and Kuyper (1945) found that Mg increased the solubility of the Ca phosphates, so that its presence seems desirable.

Technique

This was in the main as described by Cutting & McCance (1946*a*), using Barcroft's differential respirometers. Adult rats were killed by a blow on the head, newborn ones by

decapitation, the kidneys were immediately removed and placed in medium C, in which the slices were prepared. Usually these were cut free hand (Dixon, 1943), but the kidneys of some newborn rats were sliced by the method of Deutsch (1936), as modified by Cohen (1945). The slices were more easily cut by this method, but in the Barcroft flasks they seemed to lose more N, and not to maintain their respiration quite so well. The tissue (50–70 mg) was bathed in 2.7 ml of fluid in each manometer flask. The slices were roughly and quickly weighed on a glass fibre microbalance similar to the much more sensitive quartz fibre balance described by Lowry (1941). The centre tubes contained 0.3 ml. of 10% KOH and the usual roll of filter paper. The flasks were gassed with pure O₂ in the bath at 38°, a point discussed later. Readings usually commenced about 45 min after the death of the animals and were repeated every 15 min. Although physiologically objectionable, continuous removal of CO₂ is necessary in order to follow the variation of respiration with time. Warburg (1923) gave an estimate of 23 mm. Hg for the pressure of CO₂ at the centre of a slice 0.47 mm thick respiring in O₂. As the slices in this work were about 0.3 mm thick, the cells were exposed to a pressure of CO₂ which was below that in their normal surroundings (40–50 mm Hg according to Campbell, 1931), but which was not negligible. The adequacy of the buffer was checked by adding phenol red at the end of every experiment.

Nitrogen was determined by the micro Kjeldahl method. The tissue fragments were transferred to a Pyrex tube, and the fluid remaining in the flask was added to 10% tri-chloroacetic acid (1 ml) in a centrifuge tube. The tissue, the precipitate and the supernatant fluid (first evaporated to small bulk) were then separately digested for 12–24 hr with H₂SO₄ containing copper selenide. The NH₃ formed was released by steam distillation, trapped in 2% boric acid coloured with bromocresol green, and titrated with 0.0143 N H₂SO₄. The three analytical figures gave the total amount of N in the slices in each flask, and the amounts lost to the medium as 'protein' N and 'non protein' N.

RESULTS

Kidney slices from adult rats

Respiration Table 1 shows the average respiratory rates of adult rat kidney slices during the first hour

Table 1 Average respiratory rates of adult rat kidney slices during the first hour in various media at pH 7.4

Medium	Glucose	Ca	Respiratory rate	No of expts
			Mean \pm S.D. (μ l O ₂ /mg total N/hr)	
A	+	+	137 \pm 7	7
B	+	–	157 \pm 14	5
C	–	+	140 \pm 11	8
D	–	–	128 \pm 13	6

Significance of differences		
	<i>t</i>	<i>P</i>
A and B	2.8	<0.02
A and D	1.45	0.15
B and C	2.15	0.05
B and D	3.2	0.01
C and D	1.6	0.15

in the four media at pH 7.4. They only varied between 128 and 157 μ l O₂/hr/mg total N, but it will be seen that Ca tended to increase the respiratory rate, and glucose increased it considerably. The latter effect was not observed, however, when glucose was added to a medium which contained Ca. The scatter shown by the standard deviations was partly due to grouping together results obtained from the kidneys of many rats. The same effects were also observed when slices from the kidneys of the same rat were compared in the different media.

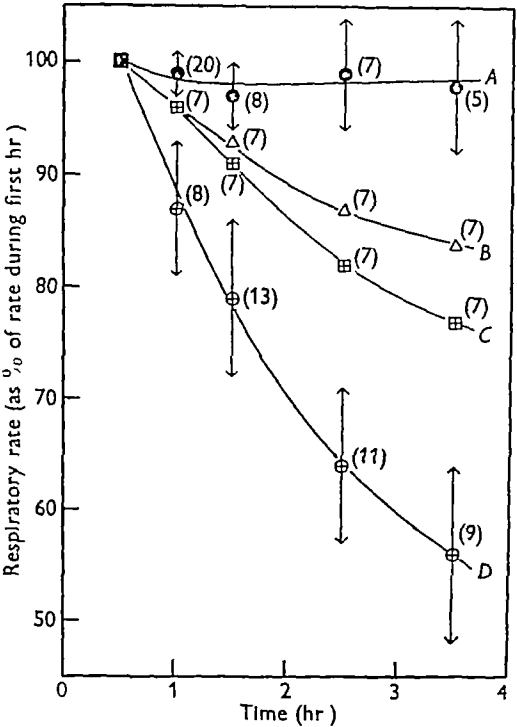


Fig. 1 Respiratory rates of adult rat kidney slices in media A, B, C and D (see p. 68, col. 2, para. 2) at pH 7.4. Average respiratory rates expressed as percentage of O₂ uptake during first hr. Arrows show standard deviations. Numbers of experiments shown in brackets.

More striking differences were revealed when the experiments were continued for 4 hr, and Fig. 1 shows how the respiratory rates fell off in the different media. The ordinates are averages of the figures obtained by expressing the O₂ uptake in a given 60 min as a percentage of that in the first hour of the same experiment. They have been plotted at the mid points of the 60 min periods, and the value of 100% for all initial periods has been plotted at 30 min. Standard deviations of the results in media A and D have been indicated by arrows, and the number of experiments from which each average was calculated is shown in brackets. It will be seen

that in the new medium, containing both glucose and Ca, there was no significant falling off in respiratory rate, for the slight decline in the averages was always less than the standard deviation. When both glucose and Ca were omitted, the respiration fell off 40–50% in 4 hr, but either glucose or Ca could reduce this decline to about 20%.

The slices still appeared normal to the naked eye after 4 hr shaking in many of the media which contained Ca, and slices which had stood in medium C for 3–4 hr at room temperature, and had then been kept in it overnight in the refrigerator at 4°, also appeared normal. In nine experiments, in which slices stored in this way were suspended in medium A in the Barcroft flasks 24 hr after they had been prepared, the average Q_{O_2} was 93% (s.d. 5) of that found for slices from the same kidneys within an hour of the death of the animal. The respiration of these slices fell off about 10% in 2 hr, which was a smaller decline than that found by Cutting & McCance (1946a) for fresh slices in their phosphate solution without Ca. Slices which had been kept overnight in solutions containing no Ca were found to have lost most of their respiratory activity.

Loss of nitrogen. Table 2 shows the percentages of N which were lost by slices of adult rat kidneys after 4 hr observation in the Barcroft flasks in

In a number of experiments, each made on slices from the kidneys of one adult rat, the losses of N were determined after different periods in the Barcroft flasks. Six manometers were prepared, gassed together in the bath, and then dismantled one at a time, starting at the end of the period of equilibration, so that the amounts of N in the slices and in the medium could be determined. Fig 2 shows the

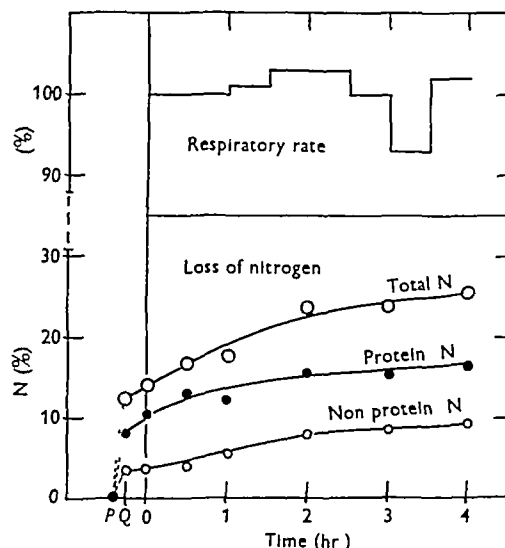


Fig 2 Adult rat kidney slices in presence of Ca and glucose, at pH 7.4. Respiratory rate in each half hour period expressed as percentage of O_2 uptake during first hr, losses of N as percentage of total amount of N originally present. P (arbitrary time scale) represents zero losses when the slices were put into the flasks. Data plotted at Q were obtained by shaking slices by hand for 5 min at room temperature.

Table 2 Average amounts of nitrogen lost by slices of adult rat kidney after respiring for 4 hr in various media at pH 7.4

(All losses are expressed as % of the total N originally present)

Medium	Glucose	Ca	No of exps	Losses of N		Total
				As 'non protein'	As 'protein'	
				Mean \pm s.d.	Mean \pm s.d.	
A	+	+	7	8.2 \pm 0.7	17.0 \pm 1.2	25.2
B	+	—	6	10.6 \pm 1.0	28.0 \pm 5.6	38.6
C	—	+	11	9.4 \pm 1.1	23.1 \pm 4.2	32.5
D	—	—	4	10.3 \pm 0.5	28.3 \pm 2.5	38.6

Significance of differences in total N lost

	<i>t</i>	<i>P</i>
A and B	9.2	<0.001
A and C	3.4	<0.01
C and D	3.0	0.01

different media at pH 7.4. It will be seen that although glucose in the absence of Ca had been found to stimulate respiration, and to reduce its decline (Table 1 and Fig 1), it had no effect upon the loss of N. Ca always reduced the loss of N, but did so more effectively when glucose was supplied in addition, and in this case the falling off in respiratory rate was abolished (Fig 1).

results of one of these experiments in medium A at pH 7.4. The respiratory rate in each half hour period is shown as a percentage of the rate during the first hour. (The apparent fall in the seventh period was probably caused by faulty re-equilibration after the manometer fluid had reached the ends of the scales.) The loss of N which had occurred by the end of the equilibration period has been plotted at zero time. The slices had then been in the flasks for about 20 min, and the shaking motor had been running for 10 min. It was assumed that no N had left the slices when they were first put into the flasks, and a point P indicating a zero loss has been plotted to the left of the origin, but not to a strict time scale. The additional data plotted at Q were obtained by shaking some slices by hand for 5 min at room temperature. As shown in Fig 2, 12.5% of the N originally present in the slices was removed in 5 min shaking, 14% had been lost before manometer readings began, and a further 11.5% was lost during the next 4 hr at a diminishing rate. Meanwhile, the

initial rate of respiration had persisted. Fig 3 shows for comparison the results of a similar experiment in which the slices were suspended in a phosphate solution actually prepared by Cutting & McCance. This time the pH was 6.8, which reduced the amount of N lost in 4 hr from 39% for a similar medium at pH 7.4 (Table 2) to 29%. This effect of pH, discovered by Cutting & McCance, made it possible to

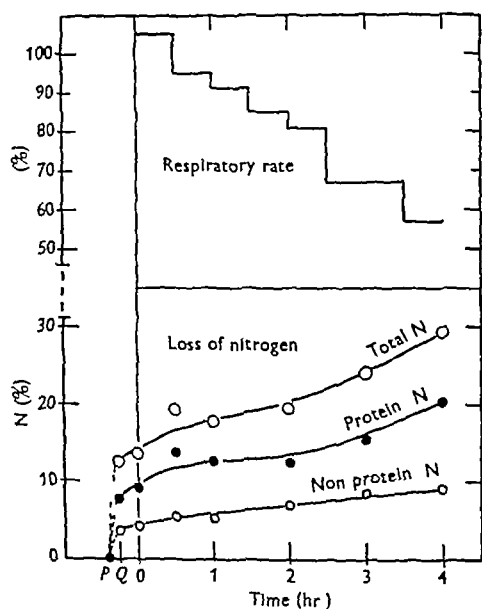


Fig 3 Adult rat kidney slices in absence of Ca and glucose, at pH 6.8. Respiratory rate in each half hour period expressed as percentage of O_2 uptake during first hr, losses of N as percentage of total amount of N originally present. P and Q as in Fig 2.

compare the loss of N in the new medium (A1) with quantitatively similar losses of N in a medium lacking Ca and glucose. It will be seen that although approximately the same total amount of N was lost in each case, the time relations were quite different. Without Ca or glucose (Fig 3) there was a loss of 14% before readings began, and another 15% was lost at an increasing rate during the next 4 hr. At the same time the respiratory rate rapidly declined, and the slices had visibly disintegrated.

Kidney slices from newborn rats

These were all studied in the new medium (A1) at pH 7.4, so that their metabolism could be compared with that of adult tissue in the same medium.

Respiration. The average respiratory rate during the first hour was found to be $106 \mu\text{l}/\text{mg}$ total N/hr (s.d. 8 in seven experiments). This is very significantly less than the figure of $137 \mu\text{l}/\text{mg}$ total N/hr given in Table 1 for adult tissue ($t=6.3$,

$P < 0.001$). The rates in the second, third and fourth hours were 96, 94 and 89% of the initial rate, with s.d.'s of 7, 10 and 13 respectively. The scatter and the tendency for the O_2 uptake to decline were both increased by the inclusion of results from slices cut by Cohen's (1945) method.

Loss of nitrogen. The average loss of N after the respiration had been measured for 4 hr was 20.3% (s.d. 1.4 in seven experiments). This was very significantly lower than the figure of 25.2% given in Table 2 for adult tissue in the same medium ($t=6.4$, $P=0.001$). Furthermore, as some of the slices had been cut by Cohen's (1945) method, this difference was probably underestimated. In an experiment of the kind illustrated in Figs 2 and 3, slices cut free hand from the kidneys of one whole litter of newborn rats were found to have lost 12% of their N before readings began, and they lost a further 5% during the next 4 hr (of 14 and 11.5% in Fig 2).

DISCUSSION

Most of the respiratory rates have been expressed in $\mu\text{l } O_2/\text{mg}$ total N/hr. The final dry weight would not have been a suitable basis for comparing the respiration of slices in the different media because they lost different amounts of material. It would have been equally unsatisfactory for comparing the respiratory rate of adult tissue with that of infant tissue, which contains a higher proportion of water. For this purpose the initial moist weight, advocated by Bach (1944), would have been no better, and nitrogen can be determined more precisely. It might appear even better to give an instantaneous rate of respiration per unit of nitrogen remaining at any moment. There is, however, no simple means of determining this, and if it had been done the tissues respiring in medium A would have been credited with an increasing respiratory rate.

Glucose was chosen as substrate because it is generally available in the body, and can serve the cells as a source of glycogen and of three carbon compounds which are not normally found outside cells in comparable concentrations. A high concentration of lactate or succinate might alter the course of the metabolic reaction chains, and give information about the quantity of one enzyme system in the cells rather than about respiration as a co-ordinated activity. According to Shipley (1944), the amounts of tissue used in these experiments could have produced about 1 mg of glucose in 4 hr, a quantity comparable with that supplied in the medium (2.7 mg), so that little effect on the oxygen uptake might have been expected to follow the addition of glucose. It was in fact found that, in the presence of calcium, glucose had no effect upon the initial respiratory rate, but the later decline in respiration and the increased loss of nitrogen which occurred

when glucose was omitted from the medium may have been related to the exhaustion of the glucose and glycogen originally present in the cells, and to the breakdown of proteins for gluconeogenesis. This response of the slices to the presence or absence of glucose is interesting in connexion with the demonstration of Jiménez-Díaz & Souto Candeira (1948) that the kidneys in the body add glucose to the blood when the blood sugar level is low, but take up glucose and store it as glycogen when the blood sugar is high.

The respiration of the adult kidney slices in the absence of glucose and calcium fell off only about half as much as Cutting & McCance (1946*a*) had found, even when a solution prepared by them was used. However, whereas they had gassed their flasks at room temperature, in this work the gassing was done in the bath at 38°, and a longer time was allowed for equilibration, so that part of the decline must have been over before readings began to be taken.

The loss of nitrogen occurred in two well defined phases. There was first a rapid loss of about 12–15% of the nitrogen originally present in the slices. This occurred during the equilibration, and it did not appear to depend upon either the composition of the medium or the age of the tissue. The nitrogen lost was probably contained in blood and cellular debris washed away when shaking began. It is unlikely that this material contributed appreciably to the uptake of oxygen, and the presence of so much inert matter constitutes an objection to calculating the respiratory rates on a basis of total nitrogen. However, it would also have been included if the initial moist weight had been determined, and with a standardized technique it did not seem to vary enough to cause serious irregularities in the respiratory rates based on total nitrogen.

A further, much slower loss of nitrogen occurred during the next 4 hr while the oxygen uptake was being measured, and in this phase the rate of loss depended on the composition of the medium and the age of the tissue. With adult kidney slices, the loss was least in the new medium containing calcium and glucose, and its rate decreased during the period of observation (Fig 2). As this loss of nitrogen was not associated with a change in the respiratory rate, the nitrogen cannot have come from a part of the cells concerned with respiration, and the loss may not have been unphysiological. Recent work using metabolites which contain unusual and recognizable isotopes has shown that the apparent permanence of cellular structures is the result of nicely adjusted dynamic equilibria. Thus Shemin & Rittenberg (1944) found that half the nitrogen in the liver proteins of the adult rat was exchanged with nitrogen outside the cells in about a week, and Schoenheimer (1942) gave a table suggesting an even more rapid turnover in the kidney of the same animal. During the fourth hour of the experiment illustrated in

Fig 2 the slices lost about 1% of their nitrogen. Had this rate continued without further decrease, the cells would have lost half their nitrogen in about 2 days, which is of the same order as the rate of exchange deduced from work with isotopes. The rate was in fact decreasing, which might be taken to suggest the approach to an equilibrium in which nitrogen could be taken up again from the medium as fast as it was being lost. The cells were in negative nitrogen balance because the medium contained at first no nitrogen for exchange, and the result was a slow loss of nitrogen unassociated with any decrease in the rate of respiration. If such an interpretation is feasible, it is interesting to look upon the slower loss of nitrogen from the infant tissue suspended in the same medium in the light of a suggestion of Rittenberg & Shemin (1947) that young animals grow, not because their cells synthesize protein more rapidly than those of adults, but because their autolytic processes are slower.

In incomplete media which did not contain calcium the second phase of the loss of nitrogen from slices of adult tissue proceeded at an increasing rate (Fig 3), and at pH 7.4 about twice as much was lost during this phase as was lost when calcium and glucose were present. It seems reasonable to attribute the additional loss of nitrogen that occurred in the absence of calcium to the breaking down of dying cells, for it was associated with a fall in respiration. The escape of protein through damaged cell membranes may also have contributed to it.

Changes in the permeability of the cell membranes may explain the influence of calcium on the initial rates of respiration in the presence and absence of glucose (Table 1). Cutting & McCance (1947) summarized the evidence that calcium tends to stimulate the respiration of the intact cells of some tissues, including rat kidney, but inhibits the oxygen uptake when it is allowed access to the intracellular enzymes by muncing or grinding the tissue. Although the increase in respiration shown in the comparison between media *D* and *C* in Table 1 did not reach the conventional level of statistical significance, similar results were reported by Kisch (1934), among others quoted by Cutting & McCance (1947). If calcium inhibits the enzymes, its action in stimulating respiration must be exerted from outside the cells, and the reduction in the permeability of their membranes would hinder the escape of substrates and coenzymes. It would also hinder the entry of substances from the surrounding medium, and this may explain the observation that calcium prevented any stimulation of the respiration by glucose. Dickens & Greville (1935) have described an analogous inhibition by calcium of the respiration of brain slices in the presence of glucose. The increased respiratory rate which was produced by glucose in the absence of calcium did not persist (Fig 1), and it

was not accompanied by a reduction in the loss of nitrogen. Glucose did, however, further reduce the nitrogen loss in the presence of calcium. One might say crudely that calcium helped to hold the cells together so that they could use energy derived from the oxidation of glucose to maintain their integrity. For in medium *A* their respiration was stable and the loss of nitrogen was minimal.

The steady respiration of slices of adult rat kidney in the new medium suggests that the falling off which was found by previous workers (and which could be reproduced by omitting glucose or calcium) was a consequence of the employment of incomplete media, and ought not to be accepted as a property of the tissue.

When the new medium was used to compare the metabolism of kidney slices from adult and newborn rats, the lower respiratory rates which Cutting & McCance (1946*a*) could demonstrate only during the first half hour of their experiments were found to persist throughout 4 hr. Cutting & McCance had found that the adult tissues lost more nitrogen than the infant ones, but this might have been due to a more rapid disintegration of the cells. The fact that in the new medium, when their respiration was stabilized and they did not obviously disintegrate, the adult tissues still lost more nitrogen confirms that Cutting & McCance were really dealing with an effect of age. A striking difference in sensitivity to the composition of the environment is also clearly shown by the fact that the adult slices could maintain a constant respiratory rate only in the complete medium, whereas Cutting & McCance had found the infant tissue to be far less exacting. It is interesting that Lasnitzki (1934-5) reported a lower Q_{O_2} , a slower decline in respiration, and a relative insensitivity to the ionic composition of the medium, when kidney slices from embryo rats taken in the last third of pregnancy were compared with the corresponding adult tissue. This 'toughness' in face of unfavourable conditions appears to be rather characteristic of the tissues of newborn animals. It may be an important property of the component cells of an organism which has not yet developed homeostatic mechanisms adequate to maintain that '*fixité du milieu intérieur*' which is required to enter upon a free adult existence.

SUMMARY

1 A stable medium buffered with phosphate (3 mM) has been devised for use with slices of surviving kidney tissue. Except for the absence of bicarbonate its composition resembles that of mammalian extracellular fluids.

2 In this medium slices of adult rat kidney maintained a constant respiratory rate for 4 hr, and could be stored for 24 hr at 4° without serious loss of respiratory activity.

3 Glucose and calcium were both essential for the steady respiration of slices of adult rat kidney. When either of these was absent the respiration fell in 4 hr to about three quarters of its initial rate, and when both were absent the respiratory rate fell to about half its initial value in the same time.

4 Slices of adult kidney in the presence of glucose and calcium lost about one eighth of their nitrogen during the equilibration period and a similar quantity during the next 4 hr. In the absence of calcium, whether glucose was present or not, they lost the same amount during equilibration, and about twice as much during the next 4 hr. Glucose without calcium did not affect the loss of nitrogen, though it augmented the nitrogen sparing effect of calcium.

5 When compared with adult tissue in the new medium containing glucose and calcium, slices of infant rat kidney showed a lower respiratory rate which persisted for 4 hr, and lost a smaller proportion of their nitrogen.

APPENDIX

Some data concerning the solubility of the phosphates of calcium

Mammalian extracellular fluids, according to Gamble (1947), contain about 1 mmol/l of HPO_4^{--} , for at pH 7.4 H_2PO_4 is almost entirely ionized as HPO_4^{--} and HPO_4^{--} in the proportions of 4:1. If the third dissociation constant of phosphoric acid is of the order of 10^{-12} (Sendroy & Hastings, 1926-7) only about 1/100,000 of the H_2PO_4 in a solution at pH 7.4 can be ionized as PO_4^{---} . Holt, La Mer & Chown (1925) estimated that about 7 p.p.m. of the inorganic phosphate of serum are present in this form, so that the concentration of PO_4^{---} is of the order of $10^{-3} \times 7 \times 10^{-6}$, or about 10^{-8} M. Holt *et al.* (1925) found that the stoichiometric solubility product for $Ca_3(PO_4)_2$ in a solution having the same salt concentrations as serum was $10^{-27.2}$, and Logan & Taylor (1937) and Kuyper (1945) have also reported values of the order of 10^{-26} (the concentrations being expressed in mol/l). The medium *A* 1 was made to contain 3×10^{-3} M P, compared with 1.3×10^{-3} M in serum and extracellular fluids. As its pH was also 7.4, its PO_4^{---} concentration may be presumed to have been about twice that of serum, i.e. about 2×10^{-8} M. Thus, with 2.5×10^{-3} M Ca^{++} , would make the ionic product $[Ca^{++}]^3 \times [PO_4^{---}]^2 = (2.5 \times 10^{-3})^3 \times (2 \times 10^{-8})^2$, of the order of 10^{-14} , so that, like extracellular fluids, the medium *A* 1 should have been highly supersaturated with respect to $Ca_3(PO_4)_2$. This salt does not, however, appear to be readily precipitated from such solutions. Holt *et al.* (1925) and Greenwald (1942) pointed out that it could be formed in one stage only by a reaction of the fifth order, and Bassett (1917) found that $Ca_3(PO_4)_2$ could not exist in contact with an aqueous phase at pH 7.4.

As in medium *A* 1 the predominant phosphate ion is HPO_4^{--} , at a concentration of approx. 2.4×10^{-3} M, the ionic product $[Ca^{++}] \times [HPO_4^{--}]$ in this medium is $2.5 \times 10^{-3} \times 2.4 \times 10^{-3} \approx 6 \times 10^{-6}$, which is also the value of the same product in media *A* 2 and *C*. This is about 10^{18} times greater than the ionic product for $Ca_3(PO_4)_2$. Shear & Kramer (1928) suggested that $CaHPO_4$ was the most likely substance to be pre-

precipitated, and that even when such solutions were apparently supersaturated with respect to $\text{Ca}_3(\text{PO}_4)_2$ they might remain stable unless precipitation of CaHPO_4 occurred. This suggests that in practice the amounts of Ca and phosphate which can be included in the same solution may be limited by the (stoichiometric) solubility product of CaHPO_4 , which, according to Shear & Kramer (1928), is of the order of 3×10^{-6} at 38° in a solution of the same ionic strength as the body fluids (the concentrations being expressed in mol/l). If this figure is accepted, the Ca-containing media, which had an ionic product of 6×10^{-6} , would seem to have been slightly supersaturated with respect to CaHPO_4 , but they were found to be stable. It was, however, found impossible

to incorporate a corresponding amount of CaCl_2 in the solution used by Cutting & McCance (1946a) at pH 7.4 without a dense precipitate forming immediately. As their solution contained 10^{-2} mol P/l, the ionic product would have been of the order of $2.5 \times 10^{-3} \times 8 \times 10^{-3}$, or 2×10^{-5} .

I wish to thank Prof R. A. McCance for suggesting the problem, for continued interest, and for invaluable assistance with the presentation of the results, Dr G. D. Greville, who kindly read the manuscript and made a number of helpful suggestions, and the Governing Body of Emmanuel College for making it financially possible for me to work in Cambridge.

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A New Manometric Method for Determination of Respiratory Quotients

BY H LASER AND LORD ROTHSCHILD

Publication of this paper is unavoidably postponed as it has not been possible for the authors to check the proofs owing to absence abroad

The Estimation of Trichloroethylene in Blood

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(Received 22 November 1948)

A review of the literature on the estimation of chloroform, carbon tetrachloride and trichloroethylene is given by Daroga & Pollard (1941) and by Habgood & Powell (1945). The methods employed depend upon (i) pyrolysis of the halogen compound and absorption of the resulting gases in alkali or alkaline reducing agents, or (ii) hydrolysis by boiling alkali—followed in each case by the estimation with silver nitrate of the halide ion produced—or (iii) the measurement of the colour formed in the upper layer on heating solutions of these substances in an inert solvent with pyridine and a solution of sodium hydroxide of such a concentration as will form a two phase system, a reaction first described by Fujiwara (1914). Of these the third has been most extensively used in recent years. Daroga & Pollard (1941), after a very thorough investigation of the conditions, developed the method for the estimation of chloroform and carbon tetrachloride in acetone solution, whilst Habgood & Powell (1945) used it to estimate these and also trichloroethylene dissolved in toluene.

Since 1944 further papers have appeared which have, however, added little to the technique of the colour development process devised by these authors. Webb, Kay & Nichols (1945) defined optimum conditions for colour development with standard acetone solutions of various halogen compounds, and recorded minimum concentrations of each which they could detect, but gave no details of their method. Rogers & Kay (1947) recorded the use of a single phase reagent for carbon tetrachloride prepared by

shaking pyridine with 15% (w/v) sodium hydroxide, separating the upper layer, and diluting somewhat with water. Colour development was brought about by heating 10 ml of this reagent with 5 ml of an acetone solution of carbon tetrachloride at 70° for 15 min. The reagent deteriorated on keeping and the method appears to offer no special advantage.

In connexion with an investigation of the use of trichloroethylene in midwifery by one of us, it was necessary to determine as accurately as possible the concentration of this drug in maternal and foetal blood when only at an analgesic level, bearing in mind that, as the volume of foetal blood available for estimation would be no more than a few millilitres, the amount of drug to be estimated would be extremely small. To this end the method of Habgood & Powell has been reinvestigated to determine its applicability, using only 1 ml blood samples at low trichloroethylene concentrations. Unexpected difficulties arose and the method of colour development has consequently been considerably modified.

Three methods have been recorded for the recovery of chlorohydrocarbons from blood for the purpose of estimation. Habgood & Powell (1945) steam-distilled the blood sample and collected trichloroethylene in toluene. Kulkarni (1944) swept out chlorohydrocarbons into ice cold pyridine by means of a stream of air, and, since the present work was concluded, Burgen (1948) has claimed considerable advantages for a method utilizing the Conway microdiffusion technique with an accuracy of $\pm 5\%$.

for chloroform, no recovery figures are quoted for trichloroethylene. We have found the steam distillation method to give excellent results with a modified form of the apparatus described by Habgood & Powell

EXPERIMENTAL

In Habgood & Powell's (1945) method of estimation, toluene extracts of trichloroethylene were heated with pyridine and 20% (w/v) NaOH. After cooling and separating the alkali the coloured solution, which was turbid, was cleared by diluting with water to 15 ml, at times it was necessary to add more water, in which case the final volume was noted and a correction made. The present work has shown that it is not always possible to disperse the turbidity completely in this way, and in any case it is doubtful whether, in a method dependent entirely on arbitrary conditions, such a variation in the composition of the solution is justifiable. Further, whereas the colour obtained was mostly the orange red described by Habgood & Powell, there appeared not infrequently a bluish red tint which persisted for several hours. Burgen (1948) has also referred to the much smaller extinction given by trichloroethylene compared with CHCl_3 , when determined by this method, though the two substances appear to give much the same colours in qualitative tests using the Fujiwara (1914) reaction. Moreover, the method also entailed the quantitative separation of the coloured layer from the alkali, a process at best of doubtful efficiency. The conditions of colour formation have therefore been reinvestigated with the following conclusions:

(1) Increase in the concentration of NaOH decreased the rate of colour formation, but eliminated the turbidity with solutions of concentration at least 7M. With 10M NaOH no colour resulted unless the two layers were mixed by shaking or stirring during heating. (2) A high ratio of pyridine to toluene, or other organic solvent, led to the rapid formation of the orange red colour, whereas a low ratio resulted in the slow formation of the blue red colour. (3) The blue red colour faded very rapidly in sunlight, but was stable in subdued daylight and in artificial light. (4) Anisole, of a limited number of solvents tried, appeared to be preferable to toluene. (5) Very high alkali concentration in the pyridine, resulting from the use of 2M tetraethylammonium hydroxide, caused a blue colour of high extinction to appear slowly in the cold. This only attained maximum intensity in about 1 hr and was rapidly changed to the orange red on gentle warming.

In accordance with these findings the method of colour development described below was adopted.

Materials

Pyridine 'Pure anhydrous' or A.R. pyridine was dried over KOH (sticks) and redistilled. Lower grade pyridine should be avoided.

Anisole was washed twice with dilute NaOH, three times with water, dried over anhydrous CaCl_2 and redistilled.

Sodium hydroxide Approx 45% (w/v) NaOH was standardized with acid and then diluted to 10M.

Tetraethylammonium hydroxide reagent was prepared by adding to 10M NaOH sufficient of the quaternary hydroxide solution to make it 0.025M with respect to that base.

Apparatus

In the early stages of the recovery of trichloroethylene from blood by steam distillation, using the apparatus of Habgood & Powell (1945), much air will inevitably escape somewhat rapidly through the absorbing liquid until thermal equilibrium is attained within, and in the present work there

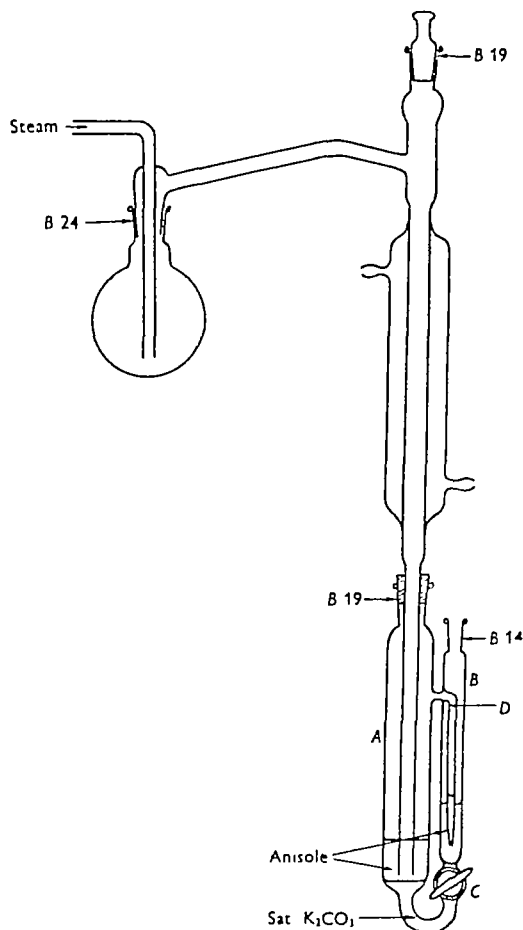


Fig 1 Apparatus for the estimation of trichloroethylene in blood

was evidence that a small amount of trichloroethylene could be lost during this period. To overcome this difficulty a somewhat more elaborate absorption apparatus has been devised and incorporated as receiver in place of the calibrated cylinder employed by Habgood & Powell (Fig 1). The dropping funnel used to contain water with which to wash down the inside of the condenser has also been replaced by a Quickfit B 19 socket and stopper. This apparatus, in which the effluent air was washed through two lots of anisole which could be mixed prior to sampling, had two chambers, A (25 mm internal diam, 20 cm long and about 90 ml capacity) joined by 6 mm tubing and a stopcock (C, 5 mm bore) to B (12 mm internal diam, 12 cm long and about 20 ml. capacity). A and B terminated in B 19 and B 14 ground sockets respectively. A side tube (D, 3 mm diam) left A at a point approx. 5 cm down the side, passed through

the wall of *B* and was bent and drawn off to a jet of 1 mm diam to form a bubbler within *B*. *A* was graduated to indicate when approx. 40 ml of distillate had collected

Method for blood analysis

The tap *C* was freed from grease and lubricated with a little Aquadag, the surplus of which was removed by running water through the apparatus whilst rotating the tap until the water discharged was no longer darkened. The dead space between *A* and *B*, comprising the whole of the 6 mm tube and the tap *C*, was filled via *B* with saturated K_2CO_3 solution and *C* was closed. The volume of this solution should not be less than 5 ml and any surplus over that required to fill the dead space was allowed to collect in *A*. The K_2CO_3 solution also ensured, by increasing the otherwise very slight difference of density between the aqueous and anisole layers, that the latter remained on top throughout the distillation and facilitated its rapid separation after mixing. Exactly 9 ml. (later reduced to 8 ml.) of anisole were run in, of which roughly 3 ml went into *B* and the rest into *A*, and the apparatus, surrounded by ice water, was then attached by a rubber stopper to the condenser of the steam distillation apparatus so that the end of the condenser dipped almost to the bottom of the anisole in *A*.

The blood sample was measured from a 1 ml blood pipette (which had been calibrated by weighing blood of known density delivered from it) into a 250 ml round flask with standard *B* 24 ground neck, and diluted with 50 ml distilled water as described by Habgood & Powell (1945). A few drops of tri *n* butyl citrate were added to control frothing and the flask attached to the distillation apparatus. The stopper at the top of the condenser was wetted with water and replaced, and steam was passed slowly through the flask until air ceased to be expelled through the solvent in *B*, after which the distillation was allowed to proceed rapidly until 40 ml of distillate had collected. The absorption apparatus was then detached and the condenser washed down inside (via the socket at the top) and outside with distilled water which was allowed to drain into *A*. *A* and *B* were stoppered, the tap *C* opened and the contents of *B* run into *A* as completely as possible, with *C* now closed the whole contents of *A* were mixed thoroughly by inversion. Part of the anisole was then transferred to *B* via *D* by loosening the stoppers and tilting the apparatus. *B* was rinsed and the anisole returned to *A* via *C* and remixed. This rinsing operation was repeated twice, and after a final mixing the two layers were allowed to separate completely in *A*.

Colour development

The anisole solution of trichloroethylene (5 ml) was added to dry colourless redistilled pyridine (5 ml) and 10M NaOH (3 ml) in a 6 in \times 4 in Pyrex test tube which was immediately placed in a boiling water bath and the mixture stirred mechanically for 10 min. at such a rate as to ensure thorough mixing of the two layers. This and subsequent operations had to be carried out in subdued daylight or in artificial light since bright sunlight caused rapid fading of the colour. The water bath was then removed and exactly 1 ml of tetra ethylammonium hydroxide reagent added to the mixture. Stirring was continued for exactly 1 min and the tube removed and thoroughly cooled. The two layers separated rapidly and the upper pyridine layer, which was invariably free from turbidity, was removed by a pipette which had

been swept out with CO_2 free air, and delivered into a 1 cm absorptiometer cell by admitting CO_2 free air to the pipette. Ten minutes prior to use, 0.5 ml of 10M NaOH had been placed in the cell and covered with a vaselined slide which was replaced after transferring the solution. The colour intensity was measured immediately with a Spekker photoelectric absorptiometer using a water cell in comparison and Ilford spectrum blue green filter no 603. The colour, which was a bright cherry red (absorption being mainly in the yellow with some general absorption in the green of the spectrum) began to fade in subdued light after about 20 min., allowing ample time for a series of readings to be taken. The first reading of a series was sometimes a trifle low.

Calibration of the absorptiometer

Standard solutions of trichloroethylene were prepared as follows. The mean weight (*W*), of anisole delivered by the 5 ml pipette reserved for this solvent was determined. A sealed thin glass ampoule containing about 80 mg of trichloroethylene, accurately weighed, was broken under a known weight of anisole in a stoppered bottle and the solution thoroughly mixed, this primary standard was then broken down in steps by weight until a solution was obtained containing exactly 0.10 mg of trichloroethylene/*W* g solution. From this, substandards were prepared by dilution by volume, using always the same pipette for both trichloroethylene solution and pure anisole. Samples (5 ml) of each substandard solution were submitted to the colour development process described above, and duplicate Spekker readings so obtained are recorded in Table 1. As these gave good agreement at all concentrations tested a more extended series was not considered necessary. The absorptiometer readings (*S*) and trichloroethylene concentration in mg/5 ml anisole (*T*) were strictly proportional up to a concentration of at least 0.10 mg/5 ml, as was shown by plotting, and obeyed the relationship $T = 0.1434S$. The values of *S* calculated from this for each value of *T* are included in the last column of Table 1.

Table 1 Calibration of Spekker photoelectric absorptiometer using standard solutions of trichloroethylene in anisole

Trichloro ethylene (mg/5 ml anisole, <i>T</i>)	Spekker readings (<i>S</i>)			
	Found			Calc from $T = 0.1434S$
	(i)	(ii)	Mean	
0.100	0.699	0.699	0.699	0.697
0.075	0.518	0.526	0.522	0.523
0.050	0.347	0.353	0.350	0.349
0.040	0.276	0.280	0.278	0.279
0.025	0.175	0.175	0.175	0.174
0.013	0.092	0.092	0.092	0.093
Nil	0.002	0.002	0.002	0.000

RESULTS

Anticipating a variation of recovery from blood samples, the efficiency of the method was first tested by distilling 1 ml of standard anisole solutions of trichloroethylene. The results, recorded in Table 2, established that the relationship $T = 0.1434S$, obtained with dry anisole standards, applied equally well to wet solutions and that recovery was essentially complete.

Table 2 *Recovery of trichloroethylene from steam-distilled standard anisole samples*

(Volume of standard taken, 0.988 ml. Volume of anisole used for absorption, 8 ml.)

Trichloroethylene in sample (mg)	Spekker readings	Trichloroethylene recovered (mg)	
			Mean
0.175	0.690	0.178	0.176
	0.673	0.174	
0.087	0.332	0.080	0.087
	0.341	0.088	
0.044	0.163	0.042	0.043
	0.169	0.044	
Nil	0.006	0.002	0.002
	0.006	0.002	
0.173*	0.675	0.174	—

* Distilled in the presence of 5 ml. blood

Table 3 records the recovery of trichloroethylene from standard solutions in blood in the preparation of which the following routine was observed

Table 3 *Recovery of trichloroethylene from standard solutions in blood*

(Volume of standard taken, 0.976 ml. Volume of anisole, 9 ml.)

Trichloroethylene concentration (mg/ml)	Spekker readings		Trichloroethylene recovery (mg/ml)		
	(i)	(ii)	(i)	(ii)	Mean
0.126	0.483	0.471	0.128	0.125	0.126
0.063	0.242	0.233	0.064	0.062	0.063
0.032	0.121	0.118	0.032	0.031	0.032
0.014	0.058	0.051	0.015	0.014	0.014

A primary standard was prepared by breaking a thin-walled ampoule containing a known weight (30–40 mg) of trichloroethylene under 250 ml of blood in a stoppered bottle which was then mechanically shaken for 8 hr to ensure uniformity. The primary standard was diluted with fresh blood in steps by weight and each newly diluted solution similarly shaken for 2 hr. Each standard was well shaken by hand immediately before sampling and any which had stood overnight were shaken mechanically for 15–20 min.

Table 4 *Recovery of trichloroethylene from blood stored over a period of 4 days*

(Volume of blood taken, 0.976 ml. Volume of anisole, 9 ml.)

Time of estimation	Trichloroethylene concentration (mg/100 ml blood)
Immediately after drawing	3.89, 3.84
3 days after drawing	3.94
4 days after drawing	3.89
Mean	3.89 ± 0.05

In preparation for future work the keeping power of standard blood solutions was also investigated, and it was shown that such solutions maintained their trichloroethylene concentration over a period of several days if kept in well stoppered bottles in a cool place. The keeping power is illustrated by the typical values obtained with a sample of blood taken from a patient receiving the drug (Table 4).

DISCUSSION

The addition of tetraethylammonium hydroxide was rendered necessary by the sensitivity of the coloured material to carbon dioxide. The amount of alkali in the pyridine anisole layer was so much reduced by the use of 10M sodium hydroxide that, in the process of transferring the coloured layer to the absorptiometer cell, sufficient carbon dioxide was absorbed to neutralize it and the blue-red colour, which is formed only in alkaline solution, changed to a pale yellow. The measures adopted to exclude carbon dioxide were taken as additional precautions for the same reason. Kulkarni (1944) has also commented similarly on the effect of acid fumes.

Since the final method depended for accuracy on the constancy of volume of the pyridine anisole layer, as did that of Daroga & Pollard (1941), it was necessary that conditions should be exactly reproduced throughout. Care was therefore taken to ensure that there could be no variation in the amount of water taken up from the alkali solution by the pyridine anisole layer by using always exactly 10M sodium hydroxide which had been checked by titration.

A somewhat simpler procedure for colour development was also investigated in which the pyridine anisole solution and alkali were not stirred during heating. This gave reproducible absorptiometer readings proportional to the trichloroethylene concentration up to about 0.05 mg/5 ml anisole, but above this the absorption varied considerably from one estimation to another and was always too large to fit the proportionality of the lower concentrations. Such solutions were invariably more blue than normal, implying that, with greater amounts of trichloroethylene, some probably escaped reaction until the addition of the tetraethylammonium hydroxide reagent, thus leading in part to the formation of the highly absorbing blue compound mentioned above. For this reason mechanical stirring was adopted.

SUMMARY

1. New conditions have been described for the quantitative production of the coloured compound from trichloroethylene and pyridine in the presence of alkali.

2 A more efficient apparatus has been described for the absorption of trichloroethylene distilled from blood in steam. The apparatus could be used for the recovery of traces of other volatile liquid compounds.

3 It has been shown that the concentration of

trichloroethylene in approximately 1 ml blood samples can be estimated to the nearest μg at concentrations between 1 and 12 mg/100 ml.

Our thanks are due to Messrs British Industrial Solvents Ltd. for the gift of tri-*n*-butyl citrate.

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Vitamin P 3

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(Received 28 January 1949)

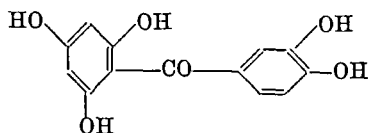
As a result of some clinical experiments on certain pathological conditions characterized by increased permeability of the capillary walls, Armentano, Bentsath, Béres, St Rusznyák & Szent Györgyi (1936) and St Rusznyák & Szent Györgyi (1936) formulated the theory of the existence of a substance called 'vitamin P' capable of increasing the resistance of the capillaries. They arrived at this conclusion because the permeability either to the whole blood (vascular haemorrhagic purpura) or to plasma protein only (various septic conditions) in their patients was decreased by treatment with extracts of Hungarian red pepper, lemon juice or fractions obtained from these extracts. On the other hand, L ascorbic acid according to them was ineffective. This hypothesis was next claimed to be substantiated by experiments on guinea pigs by Bentsath, St Rusznyák & Szent Györgyi (1936, 1937). As the data obtained with the guinea pigs did not appear convincing, the writer (Zilva, 1937*a, b*) repeated the experiments and showed that the results obtained by Szent Györgyi *et al* with these animals did not justify the interpretation placed on them. Moll (1937), Detrick, Dunn, McNamara & Hubbard (1940), McHenry & Perry (1940) and even Szent Györgyi (1938), on repetition of his own experiments, arrived at a similar conclusion.

In spite of the negative character of the above investigations a claim that a 'vitamin P' deficiency could be demonstrated by the 'capillary resistance' technique in guinea pigs was made by Zacho (1939). This procedure had been tried for a number of years by many workers to demonstrate vitamin C

deficiency in man, but no agreement had been reached as to the reliability of the test for this purpose. Zacho, however, claimed that when negative pressure was applied to the lumbar region on each side of the spine of albino guinea pigs after the removal of the hair, the pressure necessary to produce petechiae in scorbutic animals in the 'latent stage' and more so in the 'manifest stage' of scurvy, was lower than that which produced a similar response in normal guinea pigs. Furthermore, when L ascorbic acid was administered to the animals in this condition, the 'capillary resistance' rose but did not reach the normal level. The normal 'capillary resistance' could only be attained when 'citrim' (Szent Györgyi's 'vitamin P') prepared from rose hips was given either orally or intraperitoneally. Several workers (St Rusznyák & Benkó, 1941, Bacharach, Coates & Middleton, 1942, and others), also using the negative pressure technique, arrived at a conclusion similar to that of Zacho. Zacho's work called for repetition, and it is the purpose of this communication to record some representative results obtained by the writer, which demonstrate the difficulty of obtaining reproducible or significant results by these methods.

An experiment was also performed with maclurin because of the claim made by Lavollay (1944), Parrot & Lavollay (1944) and Javillier & Lavollay (1946) that a single dose of any of a number of substances not all chemically related to 'citrim', is capable of raising the 'capillary resistance' in guinea pigs as manifested by Zacho's negative pressure technique. This, according to them, is due

to the preventive action all these compounds exercise on the oxidation of adrenalin Maclurin



is one of these substances (Lavollay, 1947)

METHODS

Growing albino guinea pigs were employed in all the experiments and were kept on the following basal diet (% by wt) bran, 24, barley meal, 16, weatings, 21, fishmeal, 8, oats (crushed), 20, salt mixture, 5, water, *ad lib*. On this diet the animals develop haemorrhages and other signs characteristic of the syndrome of scurvy not longer than 15 days after being placed on the diet, and succumb to the disease 10–15 days later.

The 'capillary resistance' was assessed by measuring the lowest negative pressure required to produce petechiae. This procedure was carried out as follows. A glass tube (6 mm in internal diam), connected to a mercury manometer and a large reservoir, which in turn was connected to a water pump, was applied to the closely clipped lumbar region of the firmly fixed animals. The test area was greased with arachis oil 30 min before the test. The suction was controlled by means of a screw clip, and could be held at any desired value to within ± 0.25 cm. The negative pressure was raised in stages on a particular spot and the highest pressure maintained for 10 sec, which did not produce petechiae, was taken as the end point. After some exploratory tests, carried out on different parts of the lumbar region on both sides of the spine of each experimental animal, the actual measurements were begun a few days later, starting at a suitable spot with the pressure which previously just failed to give the reaction, and this was repeated on subsequent occasions every few days during the course of each experiment. When on repetition of the test the original spot gave a positive reaction, lower pressures were tried at different places as near as possible to the original area. It is, however, important to mention that in the same animal the lowest negative pressure required to produce petechiae varied, sometimes very considerably, not only with the side of the animal but with the place of measurement on the same side of the spine, thus yielding results of no high accuracy. It was found that temperature within the limits of 15–20° did not influence the readings.

The maclurin, which was kindly presented by Prof J. Lavollay, was dissolved in sodium bicarbonate, diluted with saline to a concentration of 1 mg/ml and injected intraperitoneally.

RESULTS

The experimental work deals with the influence on the 'capillary resistance' of guinea pigs of (1) the scorbutic diet, (2) the scorbutic diet supplemented with (a) a daily dose of 5 mg of L ascorbic acid, or (b) cabbage *ad lib*, and (3) the injection of maclurin

into animals on (a) basal diet, (b) basal diet supplemented by ascorbic acid, and (c) basal diet supplemented by cabbage *ad lib*.

In the experiments with maclurin the negative pressure measurements were made immediately before, and 2 and 24 hr after the injection of the maclurin. The measurements after the injection were carried out as near as possible to the spot used before treatment.

The figures obtained in this investigation appeared to the writer to show plainly that they did not confirm Zacho's findings, but at the suggestion of the Editor the results were submitted to a statistical analysis and the figures are incorporated in Tables 1 and 2.

Effect of scurvy producing diet and of L ascorbic acid. It will be seen from Fig 1a–f, and Table 1 (Group 1) that six animals (about 300 g body weight) on the basal diet showed only a small decline in 'capillary resistance', even after 22 days at this time the animals were suffering from well developed scurvy. In the same experiment a group of six animals received the basal diet supplemented by a daily dose of 5 mg L ascorbic acid (administered after the removal of the cabbage). These animals (Fig 1g–l, and Table 1, Group 2) gave petechiae with lower negative pressure, mostly on one side only. The statistical treatment of these results by Student's *t* test, the results of which are given in Table 1, shows that the fall in 'capillary resistance' after 21 days was just significant ($P=0.05$) in Group 2 but not in Group 1.

A third group of six guinea pigs received the basal diet plus cabbage *ad lib* for 7–9 days and thereafter the basal diet plus L ascorbic acid (5 mg/day). Over the cabbage supplemented period there was a just significant fall in 'capillary resistance', and over the ascorbic acid period of 25 days there was a rise (Table 1, Group 3), which, however, was not significant.

A fourth group of six guinea pigs received the basal diet and cabbage *ad lib* over the whole of the experimental period (7–9 days plus 25 days). These animals showed a just significant fall in 'capillary resistance' over the first part of the period, but a significant rise over the second part (Table 1, Group 4).

These results are difficult to interpret, except as a demonstration of the unreliability of the method, at least in the author's hands. However, the results claimed by Zacho are clearly contradicted—scurbutic animals showed no fall in 'capillary resistance' and administration of L ascorbic acid even produced a fall in the author's experiments, in contrast to a rise in Zacho's experiments.

Effect of maclurin. Experiments on the effect of maclurin were carried out with three guinea pigs of Group 1 and three of Group 2, a single injection being

given and observations made 2 and 24 hr afterwards. The injections were repeated after an interval of 5 days. The results are shown in Fig 1*a-c, g-i* for individual animals and in Table 2 for the groups. Another group of eleven animals (Group 5), about 200 g body weight, maintained on the basal diet with cabbage *ad lib* was similarly injected. Student's *t* test could not be applied to this case as duplicate observations have been made on the same animals.

CONCLUSIONS

According to Zacho (1939) the negative pressure required to produce petechiae in guinea pigs on a scorbutic diet falls fairly rapidly and reaches the lowest value when the animals show distinct signs of scurvy. It is evident that the above results do not confirm Zacho's observation. A particularly striking feature of these experiments is that the animals

Table 1 *Effect of diet on 'capillary resistance' of guinea pigs: statistical analysis of results*

(Measurement of negative pressures expressed as cm Hg)

Diet and description of experiment		Beginning of preliminary period 7-9 days on cabbage <i>ad lib</i>	No. of days on diet					Columns compared	<i>t</i>	<i>P</i>
			0	5	16-18	21	25			
			B	C	D	E	F			
		A								
Group 1 basal diet alone (see Fig 1)	Mean	—	34.0	32.5	30.4	26.6	—	BC	0.28	0.7-0.8
	s.d.	—	8.5	10.1	8.9	16.6	—	BD	0.72	0.4-0.5
	No. of animals	—	6	6	6	3	—	BE	0.90	0.4
Group 2 basal diet + 5 mg L-ascorbic acid daily (see Fig 1)	Mean	—	36.1	34.2	26.3	22.0	—	BC	0.42	0.6-0.7
	s.d.	—	8.4	7.2	10.9	5.7	—	BD	1.75	0.1-0.2
	No. of animals	—	6	6	6	3	—	BE*	2.56	0.02-0.05
Group 3 basal diet + 5 mg L-ascorbic acid daily (not in Fig 1)	Mean	20.0	9.6	10.8	13.7	—	14.6	AB*	3.00	0.01-0.02
	s.d.	7.6	3.9	2.8	7.9	—	4.2	BC	0.61	0.5-0.6
	No. of animals	6	6	6	6	—	6	BD	1.15	0.2-0.3
Group 4 basal diet + cabbage <i>ad lib</i>	Mean	13.8	7.9	7.3	9.3	—	10.8	AB*	2.95	0.01-0.02
	s.d.	4.1	1.5	2.3	0.7	—	1.5	AF	1.67	0.1-0.2
	No. of animals	6	6	6	6	—	6	BD	2.05	0.05-0.1
								BF†	3.38	<0.01

* Denotes significant fall. † Denotes significant rise.

Table 2 *Effect of injecting 1 mg of maclurn on the 'capillary resistance' of guinea pigs*

(Measurement of negative pressures expressed as cm Hg)

Diet and description of experiment		Before injection	2 hr after injection	24 hr after injection	Columns compared	<i>t</i>	<i>P</i>
		A	B	C			
Group 1 basal diet alone (see Fig 1) (3 animals injected twice)	Mean	27.5	36.7	39.2	—	—	—
	s.d.	9.4	8.5	8.2	—	—	—
Group 2 basal diet + 5 mg L-ascorbic acid daily (see Fig 1) (3 animals injected twice)	Mean	22.5	25.0	26.2	—	—	—
	s.d.	7.6	12.3	12.8	—	—	—
Group 5 basal diet + cabbage <i>ad lib</i> (young guinea pigs, about 200 g)	Mean	21.1	29.8	29.5	AB†	2.17	0.05
	s.d.	5.8	11.9	11.5	AC†	2.16	0.05
	No. of animals	11	11	11	—	—	—
Group 5 (same young animals injected again after 5-11 days)	Mean	17.0	19.5	20.3	AB	0.67	0.5
	s.d.	7.0	9.4	9.5	AC	0.88	0.4
	No. of animals	10	10	10	—	—	—

† Denotes significant rise.

The author, nevertheless, is of the opinion that the figures give the impression that in spite of the imperfection of the technique it would not be justifiable to dismiss the possibility that the negative pressure required to produce petechiae tended to increase after the injection of 1 mg of maclurn.

which subsisted on the basal diet alone for 22 days at a time when the characteristic syndrome of scurvy, including extensive haemorrhages, is present in a very acute form showed no significant decline in the 'capillary resistance'. Nor could a significant fall in the 'capillary resistance', similar to that

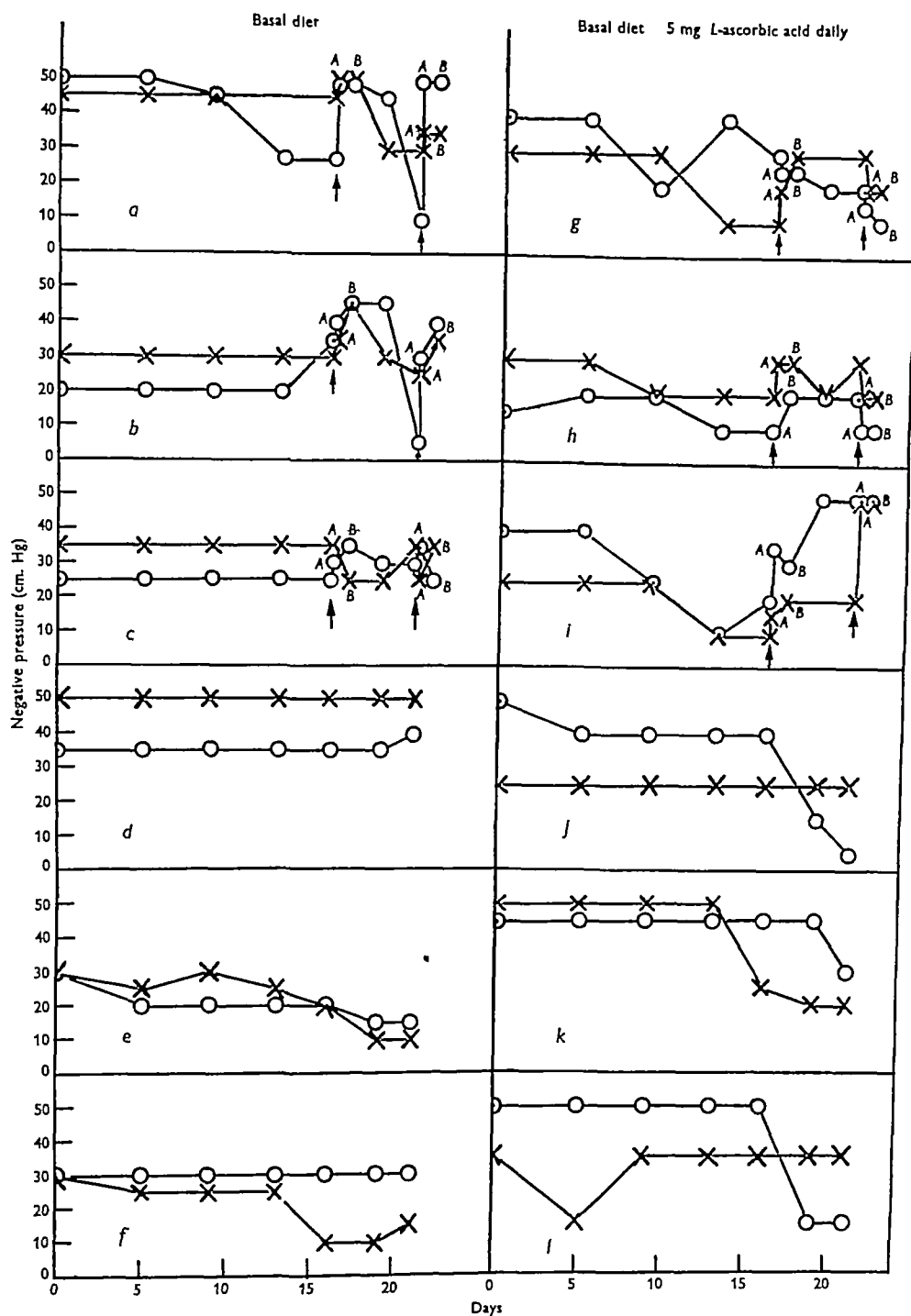


Fig 1 Effect of diet and of the injection of 1 mg of maclurin on the 'capillary resistance' of guinea pigs ↑ = injection of 1 mg of maclurin dissolved in 1 ml saline containing 0.08 ml of saturated NaHCO_3 . A = 'capillary resistance' 2 hr after injection. B = 'capillary resistance' 24 hr after injection. ○—○ = measurements on the right-hand side of the spinal cord. x—x = measurements on the left-hand side of the spinal cord.

obtained by Zacho under these conditions, be registered in the case of the guinea pigs receiving L ascorbic acid

The results of this investigation, however, suggest that after the intraperitoneal injection of 1 mg of maclurn there may be a transient rise in the 'capillary resistance'. This observation therefore tends to confirm that made by the French workers that a number of compounds, not all chemically related to 'citrin' and including maclurn and L ascorbic acid, is capable of raising the 'capillary resistance' (cf Lavollay, 1944, Javillier & Lavollay, 1946). As has already been pointed out, however, this rise occurs not only in guinea pigs subsisting on a scorbutic diet with or without L ascorbic acid but also in animals consuming a mixed diet with cabbage *ad lib*, a fact already established by Lavollay (1944). This worker, nevertheless, is of the opinion that the consumption of a scorbutic diet by guinea pigs is capable of lowering their 'capillary resistance', which can subsequently be raised by the administration of L ascorbic acid. On the other hand, he asserts that 'L'hypothèse de Szent Györgyi suivant laquelle les hémorragies du scorbut expérimental seraient spécifiquement l'effet d'une carence en vitamine P n'est pas vérifiée par l'expérience' (Lavollay, 1945). * The present results do not suggest that the cause of the rise in the 'capillary resistance' after the injection of the maclurn is due to a rectification of a dietetic deficiency but rather to a pharmacological effect (probably a direct or indirect constrictive action) of these compounds on the capillaries. A more satisfactory technique such as that used by Lee & Lee (1947), which consists of the microscopic study of the peripheral vascular system in guinea

* Prof. Lavollay informs me that since 1945 he has referred to these substances as 'substances antifrabilité vasculaire' and not as 'vitamin P'.

pigs under local anaesthesia, might throw more light on the full significance of these observations.

The above results, taken in conjunction with a critical perusal of the literature, show that there is no sound reproducible evidence which would justify the assumption of the existence of a dietetic factor essential for the maintenance of the capillary strength, the hypothetical vitamin P, the absence of which from the diet is, it is claimed, connected with the production of the syndrome of scurvy.

SUMMARY

1 The observation made by Zacho that the negative pressure necessary to produce petechiae in guinea pigs declines fairly rapidly when the animals are placed on a scorbutic diet is not confirmed.

2 Indications have been obtained that when maclurn is injected intraperitoneally into normal and scorbutic guinea pigs there is a transient rise in the negative pressure necessary to produce petechiae. It is assumed that this is solely due to a pharmacological effect and not to a rectification of a dietetic deficiency.

3 It is concluded that there is no sound evidence which would justify the assumption of the existence of an essential dietetic factor ('vitamin P') which is capable of influencing the capillary strength and that the syndrome of scurvy is produced by the absence from the diet of no factor other than L ascorbic acid.

Thanks are due to Prof. Jean Lavollay for kindly supplying me with a specimen of maclurn and for instructing me in the practice of Zacho's negative pressure technique of which he has much experience. I am grateful to various colleagues who helped me with the carrying out of the tests and with their independent reading of the results. I am also indebted to Dr W. L. M. Perry for help in the interpretation of the statistical analysis of the results.

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The Micro-estimation and Origin of Methylamine in *Mercurialis perennis* L

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(Received 31 January 1949)

The occurrence of methylamine in the tissues of higher plants appears to be very restricted. Schmidt (1878) isolated this amine from the shoots of *Mercurialis perennis* and *M. annua*. Trier (1912) reported the presence of methylamine in the rhizome of *Acorus calamus*, and Wakeman (1925) showed that it was present in the root of *Leptotaenia dissecta*. Other workers have shown that methylamine may be produced as the result of bacterial action on amino acids and nitrogenous bases. Thus Ackermann & Schutze (1910, 1911) found methylamine in cultures of *Chromobacterium prodigiosum* (*Serratia marcescens*) on potato, and Hasebroek (1887) obtained this amine from anaerobic putrefaction of choline. Emmerling & Reiser (1902) isolated methylamine from cultures of *Pseudomonas fluorescens liquefaciens* on gelatin. Guggenheim (1940) gives references to papers reporting the occurrence of methylamine in algae. In animal tissues Kohn (1931) found methylamine as an intermediate in the degradation of glycine in the liver and Ratner, Nocito & Green (1944) showed that the glycine oxidase of pig kidney catalyses the oxidation of sarcosine to methylamine and glyoxylic acid. Klein & Steiner (1928) assessed the production of methylamine in *Mercurialis annua* and *M. perennis* by means of the dimethyl α -naphthol derivative, but up to the present time a specific method for the accurate estimation of small amounts of methylamine in plant tissues does not appear to have been developed. The aim of the present work was to devise a sensitive and specific method for the estimation of very small quantities of methylamine and to study the mode of production of this base in *M. perennis* by the infiltration of possible precursors into the leaves.

EXPERIMENTAL AND RESULTS

The isolation of methylamine from tissues of Mercurialis perennis

Flowering shoots (15 kg) were harvested over a period of 17 days in batches of 1 kg, each kg was divided into portions of 50 g which were immersed in ether for 10 sec, the ether allowed to evaporate and the material minced for 5 min in a Waring blender with 100 ml distilled water acidified to pH 4.5 with 0.01N HCl. The mince was filtered through fine muslin and as much liquid as possible removed from the cell debris by squeezing. The filtrates from

each portion were combined, rapidly heated to 80° to coagulate protein and centrifuged. The supernatant was treated with excess of $Mg(OH)_2$ and steam distilled into 0.1N-HCl (25 ml) until 100 ml of distillate had passed over. This procedure was repeated until 15 kg of material had been extracted. NH_3 was removed at pH 7.4 from the combined distillates (1875 ml) by the method of François (Pugh & Quastel, 1937), the filtrate from the François separation treated with excess of $Mg(OH)_2$ and distilled into 50 ml of a solution of picrolonic acid (70 mg) in ethanol. The picrolonate solution was concentrated to small bulk and allowed to crystallize. After two recrystallizations from aqueous ethanol, 35 mg of picrolonate was obtained which melted at 242° (decomp). The melting point of an authentic specimen of methylamine picrolonate was 243° (decomp), mixed m.p., 242° (Found N, 23.50, calc. for $CH_5N \cdot C_{10}H_8O_5N_4$, N, 23.73%).

The micro-estimation of methylamine

Methylamine reacts quantitatively with ninhydrin in acid solution to give formaldehyde and NH_3 . Glycine and *N*-methylethanolamine also react with ninhydrin under similar conditions to give formaldehyde. In the case of *N*-methylethanolamine, the reaction is presumably due to the splitting off of methylamine. Alexander, Landwehr & Seligman (1945) have adapted this reaction for the estimation of glycine in blood by determining the liberated formaldehyde colorimetrically with chromotropic acid. The reaction can be made specific for methylamine, as this amine is readily volatile and can easily be separated from glycine and *N*-methylethanolamine by distillation at low temperature. *N*-methylethanolamine is slightly volatile in steam, but does not distil over if the distillation is carried out at a low temperature under reduced pressure. For the estimation of methylamine in tissues of *M. perennis*, the method of Alexander *et al.* (1945) for glycine was used with minor modifications. The intensity of the reddish violet colour given by formaldehyde with chromotropic acid was measured with the Hilger Spekker absorptiometer using the Ilford spectrum yellow filter. For the calibration curve, pure methylamine hydrochloride was used. Leaves (20–50 g) were immersed in ether for 10 sec and the ether allowed to evaporate. The leaves were minced in a Waring blender with 50–75 ml. distilled water acidified to pH 4.5 with 0.01N HCl. The mince was filtered through fine muslin, squeezed out, and the cell debris washed twice with 10 ml. distilled water. The filtrate and washings were rapidly heated to 80°, centrifuged, the clear supernatant poured off and the residue washed once with 10 ml. distilled water. The extract was transferred to a distillation flask, treated with excess of $Mg(OH)_2$ and distilled *in vacuo* at 40° into 0.01N HCl (5 ml.) until the volume was 15 ml. To concentrate the methylamine

solution further at low temperature, the microdiffusion technique of Conway & Byrne (1933) was used. Large dishes were constructed from glass capsules of 9.5 and 4.5 cm diameter. A portion (10 ml.) of the distillate was placed in the outer chamber and 1 ml 0.01N HCl in the inner vessel. Saturated K_2CO_3 (5 ml) was added to the outer chamber and the dishes incubated at 30° for 16–18 hr. The methylamine solution was transferred quantitatively to a micro distillation apparatus and the estimation carried out as for glycine (Alexander *et al.* 1945). The accuracy of the method was tested by adding a known amount of pure methylamine hydrochloride to the mince in the Waring blender. An average recovery of 95% was obtained. The method is sensitive to 3 μ g of methylamine.

Infiltration experiments

Wild plants of *M. perennis* and *Chenopodium album* L. were used, *C. vulvaria* L., *Beta vulgaris* L. and *Atropa bella donna* L. were raised from seed. Leaves (20–50 g) of *Mercurialis perennis* were infiltrated with solutions of nitrogenous bases and amino acids in an attempt to determine the source of methylamine in the tissues. Solutions of the bases (or their salts) and amino acids were neutralized (pH 7.0) where necessary and made to 0.05M. N-Methylethanolamine and dimethylethanolamine were prepared by the method of Knorr & Matthes (1898, 1901). N-Methylethanolamine and dimethylamine hydrochlorides were purified by means of the nitroso derivatives, and traces of methylamine found in the sample of trimethylamine hydrochloride were removed by treatment with nitrous acid and subsequent distillation. The vacuum infiltration method of Björkstén (1930) was employed, and, after infiltration and evaporation of excess water, the leaves were kept in a moist atmosphere in darkness for 40 hr., after which they were analysed for methylamine. In Table 1 the methylamine content of leaves of *M. perennis* removed from plants in April (plants in flower), June, July and August and infiltrated with solutions of nitrogenous bases and amino acids is given.

acids, choline, and betaines. Thus trimethylamine present in *Chenopodium vulvaria* (Dessaignes, 1852) may arise as a breakdown product of choline, and methylamine may be formed as the result of the bacterial decomposition of glycine. Although volatile amines have been reported as occurring in a wide range of plants, their concentration is generally found to be low. However, according to Kapeller, Adler & Vering (1931) trimethylamine may reach 500 mg/100 g dry weight in the red algae *Ceramium rubrum* and *Rhodomela subfusca*, and for methylamine a maximum of 40 mg/100 g dry weight was recorded in the green algae investigated. Up to the present time little or no light has been thrown on the precise mechanism of formation of methylamine and trimethylamine in plants. Decarboxylation of glycine would seem to be the simplest and most obvious reaction leading to the formation of methylamine, but a glycine decarboxylase has not yet been described (Gale, 1948) and infiltration of glycine into the leaves of *Mercurialis perennis* does not lead to a significant increase of methylamine. The experiments described above show that breakdown of methylethanolamine with the formation of methylamine takes place readily in the leaves of *M. perennis*, but they do not prove conclusively that the methylamine normally found in the tissues is produced from methylethanolamine. These experiments do suggest, however, that methylethanolamine is the precursor of methylamine even though the mode of fission is not yet known. By analogy with the behaviour of choline on heating, breakdown may take place with the formation of ethylene glycol.

In the tissues of *Chenopodium album*, *C. vulvaria*, *Beta vulgaris* and *Atropa belladonna*, methylethanolamine does not appear to be broken down with

Table 1 Methylamine content of leaves of *Mercurialis perennis* after infiltration with various possible precursors

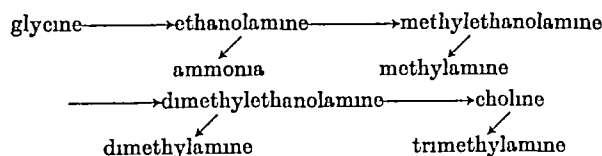
Solution infiltrated	Methylamine (μ g/100 g wet wt., to nearest μ g)						
	April				June	July	August
	(1)	(2)	(3)	Mean	(Mean value of 2 samples)		
Tap water	40	45	44	43	7	Trace	Trace
Ethanolamine	62	54	55	57	16	Trace	Trace
N-Methylethanolamine	135	146	127	136	93	76	60
Dimethylethanolamine	46	48	43	46	7	Trace	Trace
Choline (chloride)	46	50	50	49	9	Trace	Trace
Betaine (hydrochloride)	40	44	45	43	8	Trace	Trace
Dimethylamine (hydrochloride)	47	46	43	45	8	Trace	Trace
Trimethylamine (hydrochloride)	46	42	40	43	6	Trace	Trace
Glycine	53	49	50	51	12	Trace	Trace
Sarcosine	42	39	42	41	8	Trace	Trace

DISCUSSION

In living tissues the lower aliphatic amines are generally regarded as fission products of more complex nitrogenous substances such as amino

liberation of methylamine. If fission does take place in these tissues, the methylamine formed must immediately be removed. Recent work on the biosynthesis of choline summarized by Jukes (1947) has provided strong evidence for the formation of choline

by the stepwise methylation of ethanolamine, and Artom & Cornatzer (1948) have shown that administration of ethanolamine, methylethanolamine and dimethylethanolamine to rats stimulates the formation of total phospholipins. In higher plants experimental evidence in support of this mode of synthesis of choline is lacking. Attempts in this laboratory to isolate methylethanolamine from the tissues of *Mercurialis perennis* have so far failed, but there is at least presumptive evidence for its existence in this plant if the methylamine normally present is derived from it. The slight rise in methylamine content resulting from infiltration of glycine and ethanolamine may be explained on the assumption that these substances can give rise to methyl ethanolamine in the tissues. The following scheme is put forward to show possible reactions leading to the formation of the methylated amines in plants:



Choline and betaine act as methyl donors in the tissues of the rat (Stetten, 1941, Du Vigneaud,

Chandler, Simmonds, Moyer & Cohn, 1946, Du Vigneaud, Simmonds, Chandler & Cohn, 1946), but one methyl group only is labile. As a result of partial demethylation dimethylethanolamine and dimethyl glycine are formed respectively. These substances cannot act as methyl donors and their breakdown, together with that of methylethanolamine and sarcosine, involves either the splitting off of the methyl group in combination with nitrogen as dimethylamine and methylamine, or, in the case of dimethyl glycine and sarcosine, oxidative demethylation may take place with the formation of formaldehyde and glycine (Handler, Bernheim & Klein, 1941).

SUMMARY

1 Methylamine has been isolated from the tissues of *Mercurialis perennis*.

2 A sensitive and specific micromethod for the estimation of methylamine in plant tissues has been developed.

3 Infiltration of the leaves of *M. perennis* with methylethanolamine leads to a substantial increase in the content of methylamine.

4 Methylethanolamine is regarded as the probable precursor of methylamine in *M. perennis*.

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The Metabolism of the Amino Sugars

1 THE BREAKDOWN OF *N* ACETYLGLUCOSAMINE BY STRAINS OF *STREPTOCOCCUS HAEMOLYTICUS* AND OTHER STREPTOCOCCI

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(Received 18 January 1949)

The amino sugars are important constituents of many polysaccharides such as chondroitin sulphate, hyaluronic acid, chitin, heparin and salivary 'mucins', yet little is known about their metabolism. Lutwak Mann (1941) studied the breakdown of glucosamine by mammalian tissues and found that testicle, kidney and brain slice preparations oxidized the hexosamine and liberated varying amounts of ammonia. An acid substance was formed which was neither lactic nor acetic acid. Monoiodoacetate, fluorides, toluene, and merthiolate all inhibited both oxidation and deamination, whereas cyanides and glyceraldehyde were more effective against the former. This author included some preliminary results for the three bacterial strains *Escherichia coli*, *Streptococcus faecalis* and *Proteus vulgaris* and stated that the reactions differed from those carried out by animal tissues in occurring under anaerobic as well as under aerobic conditions, but that the end products formed appeared to be similar.

Very recently Rosenberg (1948) has confirmed that *Esch. coli* can deaminate glucosamine under either aerobic or anaerobic conditions in the presence of phosphate. In the absence of phosphate or in the presence of azide he claims that deamination will no longer proceed anaerobically, although dehydrogenation of the substrate still occurs.

In the present studies strains of *Strep. haemolyticus* have been examined in greater detail because these organisms might be expected to have an active amino sugar metabolism. For example, some strains form enzymes (hyaluronidases) which break down hyaluronic acid and are then able to use the liberated amino sugar (McClellan, Rogers & Hale, unpublished observation), whilst others form capsules composed of this substance (Kendall, Heidelberger & Dawson, 1937; McClellan, 1941). Streptococci of the *viridans* type have been included because it has been shown (Rogers, 1948) that the hexosamine containing substances in incubated saliva are broken down and the constituents utilized by the developing flora. Streptococci of this latter type form a very high proportion of the flora and may be responsible for the phenomenon. The streptococci have a further advantage for studying the metabolism of substituted sugars in that the products they form from glucose

are few in number, lactic acid accounting for 75–90% of the sugar used (Hewitt, 1932; Smith & Sherman, 1941), thus simplifies analytical procedures involved in comparisons with the fermentation of glucose.

METHODS

N Acetylglucosamine was synthesized from glucosamine hydrochloride by the method of Zuckerkindl & Messmer Klebermass (1931) and estimated by the Morgan & Elson (1934) procedure. NH_3 was determined by the microdiffusion method of Conway (1947), and lactic acid by ceric sulphate oxidation (Winnick, 1942), interfering substances were first removed from the samples by the method of Friedemann, Cotomo & Shaffer (1927) before making the latter estimations. Glucosamine was determined by Elson & Morgan's (1933) method and reducing sugars by that of Somogyi (1937). Nitrogen was estimated by the micro Kjeldahl technique and volatile acids by steam distillation of the sample in the presence of McIlvaine buffer at pH 2.60 (cf. Clark, 1928). At this pH no deacetylation of *N* acetylglucosamine can be detected after 20 hr. heating at 98–100° (Rogers, 1948).

Cultures of capsulated and non capsulated Lancefield Group A and Group C haemolytic streptococci and of a streptococcus of the *viridans* type were grown for 18 hr. in 500 ml. amounts of a medium consisting of 3% peptone (Evans Sons, Lescher and Webb Ltd.) and 0.2% glucose, for the haemolytic streptococci normal horse serum (5%) was included, and for the *viridans* organisms sodium glycerophosphate (2.5%) was added to prevent the pH falling lower than 6.5–6.8 (Rogers, 1945), as otherwise some inactivation of the enzyme systems occurred. After growth, the organisms were removed from the cultures by centrifuging, washed twice with M/15 phosphate, pH 7.2, and resuspended in the same buffer at such a concentration that a 1:100 dilution of the suspension in buffer caused a deflexion of 0.3 on the arbitrary scale of the Spekker photoelectric colorimeter (Adam Hilger and Watts Co. Ltd.). such suspensions were found to contain 1.0–1.2 mg. bacterial N/ml. Samples were taken from the original culture supernatants, washing fluids and final cell suspension for acid hydrolysis (2 hr. with 6N HCl) and subsequent determination of glucosamine, similar measurements were also made on the uninoculated, incubated medium. Total N was estimated on the cell suspensions.

The rate at which the various cell suspensions utilized *N* acetylglucosamine was measured by warming them to 37.5° and adding an equal volume of warm 0.02M *N* acetylglucosamine dissolved in water. Incubation of the mixtures in corked test tubes with an atmosphere of air at 37.5° was

continued until about 50% of the substrate had been used. The required period was judged by the amount of 0.1N NaOH used to keep the pH at 7.2 (cf. p. 102) using 0.001% phenol red as internal indicator. The reaction was stopped by the addition of 0.2 ml. of N HCl. The organisms were immediately removed and the supernatants neutralized to pH 7.0. *N*-Acetylglucosamine was then determined by the usual procedure in the neutralized supernatant fluids.

RESULTS

Relation between rate of utilization of N-acetylglucosamine and synthesis of glucosamine containing compounds

Table 1 illustrates the results. Strains 283 T, S 23 and E 14, which in young culture are capsulated, form considerable amounts of a glucosamine containing substance, presumably hyaluronic acid, in 18 hr cultures; it is found wholly free in the culture supernatant. The amount of glucosamine associated with

rate at which the various haemolytic streptococci utilize *N*-acetylglucosamine varies from 0.5 to 7.0 mg/hr/mg bacterial N. There is, however, no obvious relation between the ability of growing cultures to synthesize glucosamine and the rate at which washed suspensions utilize *N*-acetylglucosamine. There are insufficient results to justify calculation of correlation coefficients, nor is it thought that such an attempt would be profitable even with a more extended series until more is known of the conditions controlling synthesis. The results obtained suggest that the mechanism of synthesis of the capsular polysaccharides is unlikely to be by the direct incorporation of *N*-acetylglucosamine, initially present in the medium, since all the organisms utilize this substance rather rapidly. The rate of utilization by six out of the seven strains of the *viridans* types is very similar, and smaller than that for the haemolytic organisms.

Table 1. Rate of utilization of *N*-acetylglucosamine by washed suspensions of streptococci, and glucosamine content of cultures of the organisms

(Utilization experiments carried out at pH 7.2 and 37.5° in 0.033M phosphate buffer, cultures, 18 hr)

Organisms			Rate of utilization by suspensions (mg/hr/mg bacterial N)	Amount of glucosamine found in 18 hr cultures (mg/mg bacterial N)			
				Cells		Supernatants	
Group	Type	Strain	Exp 1	Exp 1	Exp 2	Exp 1	Exp 2
<i>Strep. haemolyticus</i>							
A	4	P 315	2.6	0.37	0.36	0	0
A	4	H 713	1.4	0.31	0.30	0	0
A	6	283 T	1.0	0.26	0.27	3.7	3.0
A	14	S 23	0.5	0.29	0.31	2.8	2.6
A	25	E 14	1.1	0.42	—	—	0.9
A	22	63 T	1.2	0.27	0.31	0	0
A	22	D 93	—	0.31	0.36	0	0
C	4	Pettman	0.9	0.34	0.36	0	0
C	20	Niel	7.0	0.30	0.25	0	0
C	—	5370	3.9	—	—	—	—
<i>Streptococci of viridans type</i>							
	1		0.5	0.31	—	—	0
	2		0.5	0.30	0.35	0	0
	3		0.75	0.30	0.30	0	0
	4		0.77	0.28	0.23	0	0
	5		0.82	0.30	—	0	0
	6		0.77	0.31	0.32	0	0
	7		—	—	0.26	0	0

0 = no increase detected in glucosamine content of supernatant compared with the uninoculated medium

the cells of both capsulated and non capsulated organisms is remarkably constant. This suggests that the substance or substances which contain glucosamine and are closely associated with the cells are altogether different from the capsular substance and may possibly form some essential building material of the cell rather than part of an extracellular polysaccharide or of a metabolic intermediate. The

The reproducibility of the figures given for the rates of utilization of *N*-acetylglucosamine was examined by testing one strain (5370) on twelve separate occasions with freshly prepared suspensions. The average rate (given in Table 1) was 3.9 and average variation of the twelve results $\pm 19.5\%$. These figures include two extreme values of 4.9 and 2.5. Other strains were also examined in a smaller

number of experiments and the results showed a similar degree of variation

The results incidently suggest that the loss of capsules in old cultures of the haemolytic streptococci is due to their solution in the culture media and not to the formation of enzymes which hydrolyse them. If the latter were the case the liberated hexosamine would have been utilized and none found in the acid hydrolysed culture supernatants

The end products from *N*-acetylglucosamine

The formation of an acid product and of NH_3 from *N* acetylglucosamine was observed in earlier experiments, qualitative tests showed that lactic acid was present. The same end products were also formed under anaerobic conditions. Estimations were therefore made of these two substances and of volatile acids, since it seemed likely that acetic acid might arise as a result of deacetylation of the amino group. The techniques used for the preparation of the cell suspensions and for following the metabolism of the amino sugar were those described above. The incubation period was extended until no further acid was formed, the pH being maintained at 7.2 as before.

Table 2 End products from *N* acetylglucosamine and from glucose by non proliferating suspensions of haemolytic and viridans streptococci

(NAG = *N* acetylglucosamine, G = glucose, 0 = none detected, — = not examined.)

Substrate	Mol/mol sugar					
	NH_3		Lactic acid		Acetic acid	
	NAG	G	NAG	G	NAG	G
<i>Strep. haemolyticus</i>						
Niel	0.97	0	1.60	1.57	—	—
283T	0.97	—	1.61	1.70	1.04	0
23N	1.00	—	1.71	1.65	1.00	0
P315	0.99	0	1.70	1.68	1.06	0
5370	1.00	—	1.60	—	1.05	—
<i>Streptococci of viridans type</i>						
3	0.96	0	1.12	1.30	1.40	—
4	0.98	0	1.33	1.36	—	—
5	0.92	0	1.44	1.42	1.10	—
6	0.94	0	1.20	1.34	1.04	—

Table 2 shows a selection of the results obtained. All the organisms yielded approximately 1 mol of ammonia, 1 equivalent of volatile acid and from 1.2 to 1.7 mol of lactic acid/mol sugar. One of the viridans strains was anomalous in forming about 1.4 equivalent of volatile acid and only 1.1 mol of lactic acid. The amount of lactic acid formed from glucose was examined in parallel experiments, as can be seen in the Table, the results are closely similar to those obtained using *N* acetylglucosamine, and agree with those already reported in the literature for the metabolism of glucose by streptococci (Smith & Sherman, 1941, Hewitt, 1932).

Carbon dioxide formation from *N* acetylglucosamine was measured for two strains only, 0.85 and 0.9 mol were formed from 1 mol of substrate. In these two examples 87 and 90 % of the carbon were recovered. Thus the end products formed from *N* acetylglucosamine appear to be closely similar to those from glucose with the addition of ammonia and acetic acid from the acetamido group of the former compound.

pH optimum for utilization of *N*-acetylglucosamine

The rates of utilization and deamination of *N* acetylglucosamine by *Strep. haemolyticus* 5370 acting at different pH's were examined as follows. A suspension of the organism was prepared in the usual manner with the exception that the final suspending fluid was water and not phosphate buffer, providing such a suspension was used immediately no detectable inactivation took place though on storage the pH fell rather rapidly and the enzyme systems were inactivated. The suspension was mixed with equal volumes of 0.2M phosphate buffers at the appropriate pH values which were checked in the presence of the cells, using the glass electrode. The mixtures were warmed to 37.5° and sufficient 0.1M *N* acetylglucosamine added to make the final concentration 0.01M. It was found that the pH drifted in those tubes in which the reaction was proceeding at optimal rate, even in the presence of the stronger buffer. Phenol red was, therefore, added to these tubes, which were within the pH range of 7.0–7.5, and the acid formed neutralized with 0.1N NaOH. When approximately half the substrate had been used in the optimal pH range the reaction was stopped by the addition of N HCl and analyses carried out as usual on neutralized supernatant fluids from the centrifuged suspensions.

Fig. 1 shows that the optimum pH for both the rates of deamination and utilization as assessed by

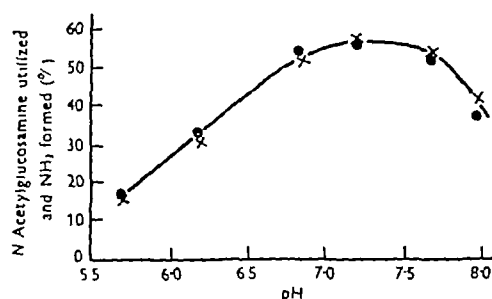


Fig. 1 Influence of pH on the metabolism of *N* acetylglucosamine by *Strep. haemolyticus* strain 5370 acting in the presence of 0.1M phosphate at 37.5°. —●— % *N* acetylglucosamine utilized, —x— % of theoretical NH_3 formed.

the usual methods is between 7.0 and 7.5. During these experiments attempts were made to use buffers other than phosphate, such, for example, as veronal (Michaëls, 1930) or acetate. The rates of both utilization and deamination, however, were about

20–30 % lower than in the presence of phosphate at the same pH. This reduction was due neither to depression by the buffers used nor to absence of K^+ ion, since the addition of phosphate but not of potassium chloride restored the rate of reaction. It is taken as preliminary evidence that phosphorylation cycles are involved in the metabolism of the hexosamine.

The optimum pH is similar to that of 7.4–7.8 recorded by Lutwak Mann (1941) for the deamination of glucosamine by animal tissue preparations. The streptococci apparently remain active at a slightly lower pH than the tissues, which were inactive at pH 6.0.

The relative rates of utilization, deamination and lactic acid formation

Lutwak Mann (1941) found that oxidation of glucosamine preceded deamination, and that utilization and deamination were not equivalent. If, in the metabolism of *N* acetylglucosamine by streptococci, oxidation at carbon atom 1 or 2 precedes deamination, *N* acetylglucosamine would disappear without the appearance of an equivalent amount of ammonia or lactic acid, for the oxidized molecule would give no colour in the usual estimation (cf Morgan, 1936; White, 1940). To examine this possibility a suspension of *Strep. haemolyticus* 5370 was prepared in the usual manner and mixed with an equal volume of 0.02M *N* acetylglucosamine. Both the suspension and the solution were first warmed to 37.5°. Samples (2.0 ml) were then taken at the times indicated in Fig. 2 and added to 0.2 ml of *N* hydrochloric acid, centrifuged immediately and the supernatant fluids neutralized. The pH of the mixture was maintained at 7.2 throughout the incubation by addition of 0.1N sodium hydroxide. Phenol red was present as internal indicator.

Fig. 2 shows that within the experimental error there is no lag in the liberation of ammonia or in the formation of lactic acid, the small difference between the curves was not a constant feature of the reaction and did not amount to more than 10 % at any point. Thus it seems unlikely that changes involving carbon atoms one or two in the *N* acetylglucosamine structure appreciably precede liberation of the amino group. In other experiments the rate of disappearance of *N* acetylglucosamine was correlated with that of total glucosamine remaining after acid hydrolysis and with that of reducing sugar. Agreement was again within experimental error.

These results suggest that any intermediate step between the unmodified *N* acetylglucosamine and the removal of the amino group is not one which is a major factor in determining the overall rate. There is no evidence for the accumulation of an amino compound. Lutwak Mann's (1941) results for tissue-slice preparations are in striking contrast. For

example, kidney and testis liberated only 20–80 % of the theoretical amount of ammonia from glucosamine irrespective of the incubation time. It seems unlikely that results for the acetylated compound would have been different since the deamination of *N* acetylglucosamine by the tissue slices was slower than that of glucosamine itself.

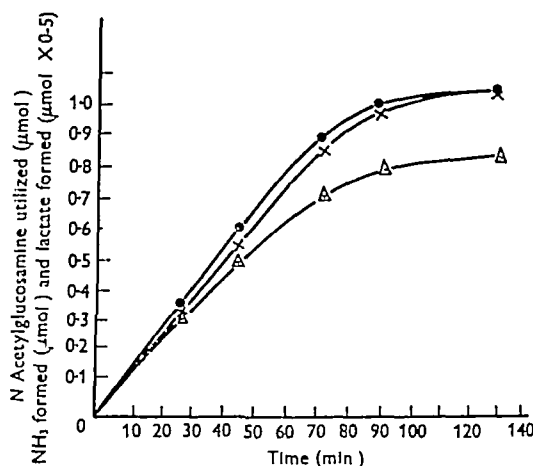


Fig. 2 Rates of utilization, deamination and lactate formation from *N* acetylglucosamine using washed suspensions of *Strep. haemolyticus* strain 5370 acting at pH 7.2 in 0.033M phosphate at 37.5°. —●— μmol *N* acetylglucosamine utilized, —x— μmol NH_4 formed, —△— 0.5 μmol lactate formed.

Inhibitors of the reaction

The influence of various known inhibitors of metabolism, on the rates of utilization and deamination of *N* acetylglucosamine and on the rate of lactate formation was examined, using *Strep. haemolyticus* 5370 as test organism. Samples of the cell suspensions, prepared in the usual way, were warmed to 37.5° and mixed with sufficient of the inhibitors to give the concentrations indicated in Table 3 after the addition of substrate. An equal volume of warm 0.02M *N* acetylglucosamine was then added. The pH was maintained at 7.2 throughout the experiment by the addition of 0.1N NaOH using phenol red as internal indicator. Incubation was continued until about 50 % of the substrate had been used in the mixtures containing no inhibitor and the reaction was then stopped by the addition of *N* HCl. Analyses were carried out on the neutralized supernatants as before.

All three stages of the reaction are inhibited by fluoride, copper ions, capryl alcohol, toluene, amino aldehyde, ketone fixatives, citrate and to a slight extent by dimedone (Table 3). Some of these substances appear to inhibit the rate of utilization rather less than they do the rate of either deamination or of lactate formation. The differences, however, are not very large and were not constant from experiment to experiment. Their significance is, therefore, doubtful. The inhibition by citrate was particularly interesting because it was later shown that glucose

also inhibited. Any possibility, however, that the mechanism of citrate action involved the four carbon acid cycle, was excluded by the negative results obtained with fumarate, malate and malonate, moreover, citrate was effective under anaerobic conditions. The inhibition was completely reversed by the addition of 0.05M magnesium chloride and therefore seems to be due to removal of either Mg^{++} or Ca^{++} ions.

Table 3 *Influence of inhibitors on utilization, deamination and lactate formation from N-acetylglucosamine by suspensions of Strep haemolyticus strain 5370*

(pH, 7.2, temp, 37.5°)

Inhibitor	Concentration (M)	Inhibition (%)		
		Utilization	Deamination	Lactate formation
NaF	0.02	83	88.5	97.0
	0.01	83	81.8	87.0
	0.001	0	17.2	12.0
Citrate	0.04	50.5	70.0	53.8
	0.02	57.9	67.8	60.5
KCN	0.01	16.8	17.5	14.6
NaHSO ₃	0.25	48.4	64.9	77
	0.12	27.9	43.2	52.8
	0.05	5.9	32.0	33.2
NH ₄ OH HCl	0.01	42.2	63.2	72.0
	0.002	27.9	18.2	22.9
	0.001	17.3	0-10	5.0
Dimedone	0.02	0-10	21.6	—
	0.01	0-10	26.2	—
	0.005	0-10	17.3	—
Capryl alcohol	Saturated solution	22.2	40.4	37.9
*Toluene	Saturated solution (a)	24.3	43.6	20
	Saturated solution (b)	100	100	100
	Saturated solution (c)	100	100	100
Amisaldehyde	Saturated solution	87.0	93.0	—
Cu ⁺⁺ as CuSO ₄ · 5H ₂ O	0.0015	22.2	17.3	39.0
	0.00015	0	4	6.0

* (a) = Toluene added to cell suspension and gently shaken immediately before experiment (b) = Toluene and cell suspension incubated together for 24 hr at normal temperature (c) = As for (b) but incubation at 37°

The degree of inhibition by bisulphite varied from batch to batch of organisms. Whereas for one batch of cells 0.02M sodium hydrogen sulphite inhibited deamination by 50-60%, with another the inhibition by a strength of 0.05M was only 30-35%. The relative efficiency of the three fixatives, bisulphite, hydroxylamine and dimedone, is also of interest and the results might most easily be interpreted by supposing that primarily they inhibit endocellular

reactions by combining with aldehyde or ketone prosthetic groups, rather than with metabolic intermediates free in the fluid. The comparative ineffectiveness of dimedone would then be explicable because cells might be expected to be less permeable to the large molecule of this substance.

The influence of glucose on the reaction

Concentrations of glucose from 0.05 to 0.2M inhibit deamination of glucosamine by 50-70% using tissue preparations (Lutwak Mann, 1941). The influence of glucose upon the utilization and deamination of N-acetylglucosamine by streptococci was examined as follows. Cell suspensions of two haemolytic streptococci (Niel and 5370) were prepared and samples (1.0 ml) distributed in tubes. Water was added to give a volume of 1.8 ml after the addition of M glucose (final glucose concentrations 0.2, 0.1, 0.05 and 0.02M). The diluted cell suspensions were warmed to 37°, and the glucose solution added, followed immediately by N-acetylglucosamine to 0.01M. The pH was maintained during subsequent incubation by the addition of 0.1N NaOH, with phenol red as indicator. Reaction was stopped by N-HCl when 50% of the full amount of acid had been produced in a tube without glucose and N-acetylglucosamine and NH₃ were determined.

Table 4 shows that concentrations of glucose from 0.05 to 0.2M inhibit both the utilization and the deamination of N-acetylglucosamine. The degree of

Table 4 *The inhibition of utilization and deamination of N-acetylglucosamine by glucose, using washed suspensions of two strains of Strep haemolyticus*

(pH, 7.2, temp, 37.5°)

Glucose concentration (M)	Inhibition (%)			
	Utilization		Deamination	
	Strain Niel	Strain 5370	Strain Niel	Strain 5370
0.2	79.0	—	83.8	93.5
0.1	79.5	75.9	67.5	71.2
0.05	54.0	49.6	50.2	62.2
0.02	29.6	4	23.6	5.5

inhibition is similar to that recorded by Lutwak Mann (1941) for tissues acting upon glucosamine. One of the interesting results obtained with tissues was that the end products formed from glucose by testicular preparations also inhibited the deamination of the amino sugar. Streptococci, however, were not inhibited by the neutralized products formed from glucose, lactate itself was equally ineffective.

Relative rates of acid formation from glucose and N-acetylglucosamine

The rates, in two strains of *Strep haemolyticus*, were measured by the amounts of 0.1N NaOH necessary to maintain a pH of 7.2. The technique of preparing the cell suspensions and of conducting the

reactions was as described for earlier experiments. The concentration of both substrates at the beginning of the experiment was 0.01M. Fig. 3 shows that the rate of acid formation from glucose is very much faster than from *N*-acetylglucosamine. There is again no evidence of lag using *N*-acetylglucosamine.

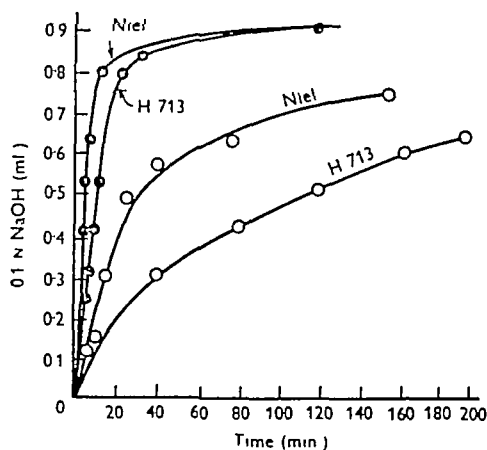


Fig. 3. Relative rates of acid formation from *N*-acetylglucosamine and glucose by washed suspensions of two strains of *Strep. haemolyticus* (Niel and H 713) acting at pH 7.2 in 0.033M phosphate at 37.5°C. —●— acid formation from glucose, —○— acid formation from *N*-acetylglucosamine.

The initial rates of the reaction were measured for nine strains of *Strep. haemolyticus* and seven of the *viridans* organisms. The rate of acid formation from glucose was always found to be faster than from the amino sugar, the ratios of the initial rates being from 2 to 20 for the former group of organisms and from 2 to 9 for the latter.

DISCUSSION

The end products formed by streptococci acting upon *N*-acetylglucosamine differ from those formed from glucose, by the additional presence of ammonia and volatile acid, but the amount of lactic acid is closely similar for the two substrates. The question is at what point or points in the fermentation is the acetamido group decomposed to ammonia and free acetic acid. One possible scheme for the breakdown of *N*-acetylglucosamine which would account for the end products obtained is deacetylation followed by hydrolysis of the amino group. The remaining glucose could then be fermented by the usual processes. The effect of inhibitors upon the reaction, however, does not favour this hypothesis, as the substances examined inhibited the formation of lactate and ammonia to about the same extent. Thus it seems that glycolysis and deamination are closely related. Otherwise we must assume that the sensitivity of the 'glucosamine deaminase' is exactly

the same as that of the various glycolytic enzymes, since inhibitors as widely apart in their mechanism of action as fluorides or citrate (which inhibit by removal of Mg^{++}), ketone and aldehyde fixatives such as bisulphite, iodoacetate (which effects enzymes depending upon thiol groups), and glucose (which presumably inhibits competitively) all influence deamination and lactate formation to about the same extent.

The way in which the glycolytic and deamination reactions are interrelated cannot be decided from the results so far obtained. The most likely hypothesis, however, on the present results and those obtained by Lutwak Mann (1941) for mammalian tissue slice preparations would seem to be that partial breakdown of the glucopyranose structure occurs before the amino group is liberated as ammonia. Although no evidence for the formation of intermediary amino compounds was obtained in the present work—the somewhat greater degree of inhibition of deamination as compared with utilization obtained using some inhibitors being too small and irregular to use as evidence—the observations of Lutwak Mann (1941) that only 20–60% of the theoretical amounts of ammonia are liberated using testicular preparations could be explained in this way. No lactic acid is formed by this tissue, although the amino group remains free throughout the reaction. It appears that here some acid amino compound must be formed, and that the testicular preparations lack the enzyme systems, which can convert it to lactic acid. The deaminase in streptococci must be very active, assuming intermediate amino compounds are formed, since there is no lag between the disappearance of the hexosamine and the appearance of ammonia. Whatever reactions are pace setting, they must leave sufficient of the *N*-acetylglucosamine molecule intact to give the colour reaction.

The other possible type of interrelation between glycolysis and deamination is that, as with glutamine (McIlwain, 1946), fermentation must be proceeding before deamination can take place. The inhibitory properties of glucose, however, do not support the idea. Using whole cells, however, permeability factors complicate the interpretation of results, and the problem would be better reserved for study with cell free enzymes. The possibility that acid amino compounds are formed during the breakdown of hexosamines suggests that an amino acid may be formed. Streptococci, however, have very limited potentialities in breaking down these substances. Hills (1940) examined washed suspensions of four strains of *Strep. haemolyticus*, Groups A and B, and one of *Strep. viridans*. He found that of the common amino acids only arginine was deaminated rapidly. Clearly the involvement of arginine itself as an intermediate in the breakdown of *N*-acetylglucosamine is impossible from both structural con-

siderations and the nature of the end products, all the ammonia was liberated from the hexosamines, whereas two amino groups would remain fixed in ornithine if arginine were an intermediate. Nevertheless, the extremely poor array of deaminases present in the streptococci renders desirable a further examination of the relations between arginine dihydrolase and the deaminase active in the breakdown of the amino sugars.

SUMMARY

1 The rates at which suspensions of *Streptococcus haemolyticus* and *Strep viridans* utilize *N*-acetylglucosamine show no obvious correlation with the amount of hexosamine synthesized by cultures

2 The end products formed by streptococci from 1 mol of *N*-acetylglucosamine are 1 mol of ammonia, 1.2–1.7 mol of lactate and 1 equivalent of volatile acid

3 The pH optimum for the reaction is 7.2–7.5

4 The rates of utilization, deamination, and lactate formation are equal

5 Reagents inhibiting lactate formation also inhibit deamination and the disappearance of *N*-acetylglucosamine

6 The most likely mechanism for the reaction appears to be the breakdown of the glucopyranose structure with the subsequent deamination of an intermediate amino compound

The author acknowledges the very considerable technical assistance given by Mr A. Parkinson during this work, and thanks Dr Nuala Crowley for the strains of *Strep haemolyticus*

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The Kinetics and Specificities of Deamination of Nitrogenous Compounds by X-radiation

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(Received 31 January 1949)

In continuation of previous investigations on the specificity of radiation effects (Dale, 1942, 1947, Allsopp, 1944, 1948) we have found that deamination occurs when aqueous solutions of amino acids are irradiated by X rays (Dale & Davies, 1949), a fact which was independently observed by Stein & Weiss (1948). The specificity of this reaction and its peculiar kinetics described in this paper are of consequence with respect to the mode of the indirect action of radiation on solutions and probably for the radiobiology of cells. An attempt will be made to interpret the experimental results in terms of the primary formation of radicals from water by X rays (Weiss,

1944). It is, however, possible that the current concept of radical formation may need modification in view of certain still unexplained facts, for example, the variation of the protective power of certain substances with their concentration (Dale, Davies & Meredith, 1949).

The approach to the problem of the relation between radiation effect and molecular structure of the solute, presented in this paper, is different from that used previously. Whereas then an overall picture of the radiosensitivity of a substance was indirectly obtained by measuring the competition between two solutes for the available radiation

energy, there is now only one solute present in any one experiment and the formation of ammonia is directly measured in relation to the molecules from which it is derived

EXPERIMENTAL

Methods Radiation was obtained either from a 500 kV Metropolitan Vickers continuously evacuated X ray tube (half value layer 5.2 mm Cu, average $\lambda = 0.056 \text{ \AA}$) or from a Victor KX 10, 140 kV tube (half value layer 3 mm Al, average $\lambda = 0.29 \text{ \AA}$). The dosage rate at the position of the solution was determined by a 250 r Victoreen condenser dosimeter which had been calibrated at the National Physical Laboratory. A Hammer dosimeter was used to integrate the total dose given. The general treatment of glassware complied with rules described earlier (Dale, Gray & Meredith, 1949). All solutions were made up by weight with air saturated water redistilled from alkaline KMnO_4 in an all glass (Pyrex) still and then exposed to radiation in fused glass ampoules.

Analytical procedure The NH_3 was distilled in a standard microdiffusion apparatus (Conway, 1947) from a solution half saturated with respect to K_2CO_3 into standard acid. Usually 1.0 ml of 0.02N H_2SO_4 was employed. For the final colorimetric estimation of NH_3 by nesslerization a Spekker photoelectric absorptiometer calibrated for the range of 1–10 μg of NH_3/ml was used. We are indebted to Adam Hilger and Co. Ltd for the loan of this instrument. Whenever a greater amount of NH_3 was found in preliminary experiments, the solution under test was immediately diluted with nesslerized water to a final concentration falling within the prescribed range. Dilution was carried out by adding rapidly the required volume of nesslerized water to the distillate contained in a test tube and completing the mixing by pouring the combined fluids back. This procedure helped to avoid interfering turbidity. Only in the few cases in which the solution was strongly alkaline (carboxypeptidase, guanidine carbonate and glyoxaline), was irradiation carried out in the Conway unit itself, the outer ring containing the solution and the central compartment the standard acid. No further alkali was added.

For all experiments blanks were included in order to determine any extraneous traces of NH_3 or any NH_3 which might have been split off from the substances under the conditions of the analysis. Any such blank value was subtracted from the experimental value. Very careful and thorough recrystallization of the amino acids, etc., is necessary in order to reduce the blanks to negligible values. In a few cases the amounts of amino acids available for the experiments were not sufficient to permit of a thorough recrystallization and such samples tended to show a high blank.

RESULTS

Variation of ionic yield of glycine with concentration

The X-ray dose for all concentrations was 166,000 r for aqueous solutions up to near saturation ($2 \times 10^5 \mu\text{g}/\text{ml}$) (The density of this solution is 1.08 and of dry glycine 1.16). The experimental points are shown in Fig. 1. They fall approximately on a curve given by $I \propto C^n$, where $n = 0.3$, I = ionic yield, defined in this paper as the number of molecules of

ammonia liberated for every 32.5 eV of X ray energy adsorbed, and C = concentration of glycine. The curve drawn in the figure is calculated on a theoretical basis (see Discussion and Appendix).

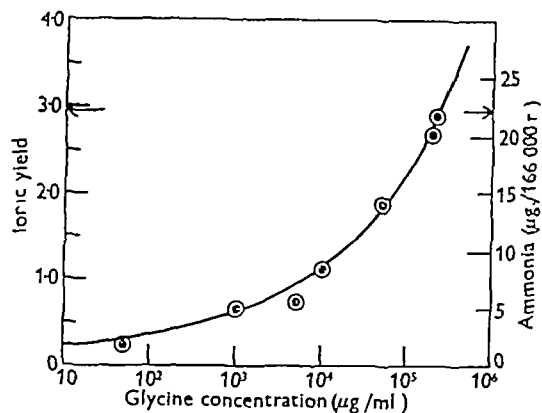


Fig. 1. Ionic and absolute ammonia yield from irradiation of glycine by 166,000 r. Points, experimental, curve, theoretical, arrow, dry glycine.

The arrow in Fig. 1 indicates the yield for dry glycine (2.96). To avoid any loss of ammonia in this case, irradiation took place in a closed system consisting of two glass bulbs fused to a connecting tube which separated the dry glycine from 0.01N H_2SO_4 . After irradiation the contents were mixed and the ammonia determined.

Table 1. Ammonia yield from amino acids and other nitrogen-containing compounds in 0.13M solution after exposure to an X ray dose of 166,000 r.

Substance	$\mu\text{g NH}_3/\text{ml}$
Glycine	8.1
Glycine + 1 equiv. HCl	5.0
Glycine + 18 equiv. NaOH	8.0
Alanine	7.2
β -Alanine	4.3
Histidine	11.8
Histidine monohydrochloride	10.6
Lysine monohydrochloride	3.7
Arginine	7.5
Cystine + 1 equiv. HCl	6.3*
Cystine + 1 equiv. NaOH	19.0*
Methionine	6.6†
Glycine anhydride	1.3
Glyoxaline	3.0
Glycylglycine	12.5
Glycylglycine + HCl	3.8
Leucylglycine	6.6
Diglycylglycine	7.0
Proline	0
Urea	0.2
Guanidine carbonate	0
Thiourea	1.4
[Carboxypeptidase‡, 2.3%, $6.6 \times 10^{-4} \text{ M}$]	9.1*

* Large blank.

† Relatively large blank.

‡ Mol. wt. 32,000 (Putnam & Neurath, 1946), 34,000 (Dale, Gray & Meredith, 1949).

*Deamination of amino-acids and other
nitrogen-containing compounds*

All compounds were irradiated in 0.13M solutions (equivalent to 10 mg of glycine/ml) with an X ray dose of 166,000 r. The results are summarized in Table I and considered in the discussion.

Effect of exclusion of oxygen

Experiments were carried out with oxygen free solutions of glycine which gave the same yield of ammonia as aerated solutions. In addition, glycine dissolved in solutions of 0.0005–0.002M hydrogen peroxide gave the same yield of ammonia as glycine dissolved in water (0.0005M hydrogen peroxide solution is equivalent to a concentration of peroxide resulting from an X ray dose of 166,000 r, if it were formed with an ionic yield of unity).

DISCUSSION

In discussing the results and attempting to fit them into current concepts of the mode of action of ionizing radiations, attention must be paid to the kinetics of the reaction and to the specificity of the radiation effects. The inactivation of enzymes (Dale, Meredith & Tweedie, 1943, Dale, 1943) and viruses (Lea, Smith, Holmes & Markham, 1944) by increasing doses of radiation was shown to follow an exponential course owing to the increasing protection given by the steadily growing fraction of solute, already deactivated, but still capable of reacting with the agent of indirect action formed from water. The radiation effect could only be measured in terms of a change of the initial concentration of the solute, a change which had to become fairly large in order to obtain accurate results. In the present investigation the ammonia split off by radiation is directly measured when only a few parts per thousand of the initial glycine have been changed. One would expect therefore an effect proportional to the dose, and this was verified experimentally.

The ionic yield of the deamination of glycine

The radiation producing deamination of dissolved glycine acts partly directly on the glycine and partly indirectly via the water. The action on dry glycine is direct only and the direct action in solution is proportional to the concentration of the solute. We can apportion the contributions of the direct and indirect action to the ammonia yield in the 18% solution, the direct action will give 3.8 μg /ml for 166,000 r, being the yield from 1.8 g of glycine, and the indirect action will therefore contribute 19.9 μg /ml to give the total ammonia yield of 23.7 μg /ml. In more dilute solutions the proportion due to direct action is even less. These considerations show that the effects are mainly indirect. The experi-

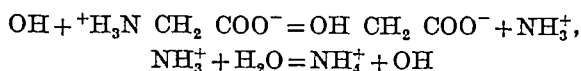
mental results have, therefore, to be discussed in terms of indirect action, the most characteristic feature of which has hitherto been the constancy of the ionic yield when the concentrations of the solute are varied, except at extreme dilutions. The deamination of glycine (Fig. 1) shows no such constancy over the whole range of concentrations from very dilute to nearly saturated solutions, indicating a more complicated mechanism. The usual explanation for the falling ionic yield at extreme dilution is the recombination of the hydroxyl radicals and hydrogen atoms formed from the water. This explanation may hold for deamination, if recombination is still appreciable at the high concentrations, because of a low affinity of the radicals for glycine (Dale, 1942).

The experiments show that the ionic yield rises to a value of 2.9 without any tendency to approach a limit. The ionic yields so far observed for biological substances lie between the limits of 0.1 and 1.0, and thus the deamination of glycine by X rays seems to be the first example of a non polymerizing reaction of an organic compound which can greatly exceed unity. (A second example is the liberation of sulphur from thiourea by radiation (Dale & Davies, 1949).)

How then can the high ionic yield both for aqueous solutions and dry glycine be explained? We shall examine two alternative mechanisms to account for it in aqueous solution, one invoking formation of several pairs of radicals per ion pair and the other a chain reaction. It is obvious that the mechanisms to be discussed for the aqueous solutions cannot explain the yield from the dry glycine, and we shall not attempt to specify the way the energy is utilized in this case.

To begin with the non chain radical mechanism Weiss (1944) has suggested that the effects of ionizing radiations are due to hydrogen and hydroxyl radicals formed by the splitting of water molecules. It has frequently been assumed that about one effective radical pair is formed for each ion pair (Lea, 1947). On the basis of this assumption an ionic yield exceeding unity could not primarily occur, since one radical by its very nature cannot react with more than one molecule of glycine. However, radicals may be formed by excitation or direct dissociation as well as by neutralization of charged ions (Danton, 1948, Miller, 1948). Miller suggests that, in the case of his inorganic solute, a total of about three pairs of radicals are formed per ion pair (or, more exactly, per 32.5 eV of energy absorbed from the radiation). This is energetically possible, since it requires 5 eV to form a pair of radicals and from the 32.5 eV absorbed only about 12 eV is needed to ionize the water molecules. If both radicals are effective (Dale, 1947, p. 49) in reacting with the solute either directly or indirectly by a mechanism such as suggested by Miller (1948), then an ionic yield as high as 6 could be obtained without any form of chain reaction.

The other alternative is a chain reaction. Dainton (1948) has recently shown that hydroxyl and hydrogen radicals catalyse the polymerization of vinyl cyanide and 2-cyanoprop-1-ene. In this reaction the radical is initiating a chain reaction which can continue without further participation of a radical of the same type. For the deamination of glycine, however, it is unlikely that after a hydroxyl radical has collided with a glycine molecule an ammonia formation could progress on its own without further radicals taking part. A mechanism on the following lines might be suggested



The re-formed hydroxyl radical can then collide with another glycine molecule and so carry on the chain.

For the deamination of amino acids by ultra-violet light (Weizmann, Bergmann & Hirschberg, 1936) an ionic mechanism was proposed on account of a maximum quantum efficiency for the isoelectric state of the amino acid examined. This mechanism does not seem to apply to the deamination of amino acids by X-rays since the hydrochlorides of amino acids yield less ammonia than the amino acids themselves (Table 1).

The variation of ionic yield with concentration of solute

The schemes suggested above give a broad picture of how ionic yields exceeding unity may occur. They do not yet account for the particular form of the variation of yield with concentration which can be explained by considering the kinetics of the reaction and the spatial distribution of the radicals. The energy of the X-radiation is transferred to electrons (Compton recoil or photoelectrons), and the radicals are formed initially along their tracks, either from ions or from water molecules directly excited or dissociated by the electron. The initial density of the radicals is high, and therefore only at high concentrations of solute will their recombination be negligible. The rate of loss of radicals can be represented by the equation

$$-\frac{dn}{dt} = -D \nabla^2 n + \alpha n^2 + \beta Cn,$$

where n is the concentration of radicals, C the concentration of the solute, D the diffusion constant of the radicals, α the velocity constant of the recombination of the radicals, β the velocity constant for the interaction of the radicals and solute leading to the removal of the radical and t the time.

At the dose rates used the highly localized formation of radicals along the electron paths confines the entire reaction to their immediate neighbourhood, so that in the equation above we need only consider

one electron track, and therefore the reaction is independent of dose rate. The initial concentration distribution of radicals has a definite value n_0 , and their subsequent fate is described by the solution of the equation. On the basis of this equation a curve covering both mechanisms has been calculated (see Appendix), the numerical values of the parameters having been chosen to give the best fit to the experimental points (Fig. 1). It is worth noting that the choice of constants is not critical and in particular that no reliable estimate can be made, in the case of the non-chain reaction, of the number of effective radicals per ion pair and in the other case of the chain length.

The peculiarity of the deamination reaction by X-rays is brought out on comparing this reaction with the inactivation of enzymes and viruses (Dale, 1947; Lea, 1946), where the recombination of radicals becomes negligible at much lower concentrations, resulting in a constant ionic yield over an appreciable range of concentrations. The deamination, in fact, never attains constancy of its ionic yield, and it is uncertain whether such a state would be reached if higher concentrations were physically possible. This uncertainty makes a rational interpretation more difficult and one can only tentatively interpret the different behaviour of the various substances being deaminated in terms of their respective affinity to radicals (considerably lower than for enzymes). This difficulty becomes even greater in the liberation of sulphur from thiourea (Dale & Davies, 1949) which follows a similar course, although protection experiments suggest a high affinity (Dale, 1947).

In the present state of knowledge one is justified in attempting an interpretation in terms of radicals and their recombination, but, as pointed out in the introduction, present views of the mechanism discussed in this paper may have to be modified in the future.

The specificity of the deamination of amino-acids and other nitrogen containing compounds

As is shown in Table 1 the α amino acids—glycine, alanine and arginine—are deaminated to nearly the same extent, whereas histidine exceeds the average value. This larger value for histidine may either be due to a contribution from the glyoxaline part of the molecule, since glyoxaline itself yields a certain amount of ammonia on irradiation, or to a weakening of the strength of the C-N bond in the α amino group through the vicinity of the glyoxaline or to both these causes. An amino group in the β position (β alanine) appears to be more resistant to deamination. In the case of cystine and methionine, deamination takes place, but no further conclusions are drawn since the blank values were too high. When an equivalent amount of alkali is added to

glycine deamination remains at the normal level, however, a most striking effect on deamination is observed when an equivalent amount of acid is added to the solution of amino acids and dipeptides. The ammonia yield then falls by about one third. Apparently the vicinity of the undissociated carboxyl group, which in the dissociated state shows a marked resistance to radiation (Dale, 1947), affects the strength of the C-N bond. The effect is masked in the case of histidine where the hydrochloric acid combines mainly with the basic group of the glyoxaline. The combination of two glycine molecules in ring form (glycine anhydride) considerably reduces the ammonia value, and similarly the peptide linkage in leucylglycine and diglycylglycine does not contribute to deamination, but glycylglycine appears to be an exception yielding more ammonia than glycine. The imino group in proline and the amino groups, combined with carbon dioxide in guanidine carbonate or free in urea and thiourea, are either completely inert or very nearly so.

In the experiments with crystalline carboxy peptidase, representing a highly purified protein, large blanks were obtained and the value of $9.1 \mu\text{g}$ of NH_3/ml given in Table 1 is therefore not very reliable.

Biological significance

From the biological point of view the high ionic yield of deamination may be of importance. The deamination of amino acids and proteins in vital regions is likely to have a toxic effect on cell functions and their coordination. It has been shown (Auerbach & Robson, 1947; Lobashov, 1937) that ammonia vapour has a slight, but definite, mutational effect in *Drosophila*. The permeation of ammonia from outside to vital centres can only be incompletely achieved and the concentrations used experimentally are limited by toxic effects on the whole organism. Radiation, however, will generate ammonia in these centres themselves and thereby produce a higher concentration than can possibly be achieved by diffusion from outside.

APPENDIX*

Calculation of ionic yields

In calculating the ionic yield of the deamination process we need only take account of radicals formed along a single electron track. We consider one type of radical only and suppose that they diffuse

* Note added in proof. Dr C. B. Allsopp recently sent us a manuscript by the late Dr Lea and himself, drafted in 1944, arriving at identical conclusions. The paper was not published because of insufficient experimental data at that time, but formed the basis of Fig. 4, p. 58, in Lea's book (Lea, 1946). We thank Dr Allsopp for encouraging us to publish our appendix in its present form.

away from their positions of formation until they are removed from the solution by either recombination with another radical or by interaction with the solute. We suppose that the radicals are initially distributed in a column along the electron tracks in a manner similar to the charged ions so that the initial density of radicals at distance r from the axis of the column is given by the following equation (Jaffé, 1913; Kara-Michailova & Lea, 1940; Lea, 1947)

$$n = \frac{N_0}{\pi b^2} e^{-r^2/b^2},$$

where N_0 is the total number of radicals per unit length of track, and b a constant giving a measure of the initial radius of the distribution.

The rate of loss of concentration of radicals is given by

$$-\frac{dn}{dt} = -D \nabla^2 n + \alpha n^2 + \beta Cn,$$

where C , D , α and β have the meaning as defined

above, $\beta = \frac{Zp}{M}$, where Z is the collision frequency

between a radical and a solute molecule, p is the probability that a reaction occurs at any collision between radical and solute, and M is the average chain length, so that there is a chance of $1/M$ that the radical is lost per encounter, for a non-chain reaction $M=1$. If diffusion only takes place, the distribution of radicals remains gaussian, and we make the assumption, following Jaffé (1913), that the distribution also remains gaussian when loss of radicals occurs due to recombination and capture, though this distribution is not an accurate solution of the differential equation. Writing, therefore,

$$n = \frac{N}{\pi (4Dt + b^2)} e^{-r^2/(4Dt + b^2)},$$

where N is the total number of radicals per unit length of track remaining at time t , we can substitute this value and integrate over the column to get

$$-\frac{dN}{dt} = \int_0^\infty (\alpha n^2 + \beta Cn) 2\pi r dr = \frac{\alpha N^2}{2\pi (4Dt + b^2)} + \beta CN$$

The total number of molecules of ammonia formed per unit length of track will be

$$p_1 \int_0^\infty ZpCN dt,$$

where p_1 is the probability of liberation of ammonia following reaction with a radical.

If we take the initial number of ion pairs per unit length of track to be N_0/R (i.e. R radicals per ion pair) then the ionic yield I is given by

$$I = p_1 R \int_0^\infty ZpC \frac{N}{N_0} dt$$

We now have to solve N as a function of time

We have so far considered one type of radical only, but if two radicals (H and OH) are present, both of which react with the solute, the same equations are obtained, if we assume that the total numbers of both types decrease at the same rate, though they may have different spatial distributions. For the constants D and b^2 we now use the mean values of those for each radical and for α the total velocity constant for recombination by all processes.

To solve the equations we make the following changes of variables

$$y = N/N_0, \quad x = \frac{4D}{b^2} t + 1,$$

and write
$$P = \frac{\alpha N_0}{8\pi D},$$

and
$$Q = \frac{\beta b^2}{4D} \quad C = \frac{1}{M} \frac{Zpb^2}{4D} C$$

The differential equation then becomes

$$-\frac{dy}{dx} = P \frac{y^2}{x} + Qy,$$

with the initial condition $y=1$ when $x=1$, and

$$I = p_1 RMQ \int_1^\infty y dx$$

The differential equation is a Riccati equation (see Piaggio, 1933) and can be solved by making the substitution

$$y = \frac{x}{Pu} \frac{du}{dx},$$

giving
$$\frac{d^2u}{dx^2} + \left(\frac{1}{x} + Q\right) \frac{du}{dx} = 0$$

This gives
$$\frac{du}{dx} = \frac{A}{x} e^{-Qx},$$

where A is a constant, and therefore

$$u = A \int_1^x e^{-Qx} \frac{dx}{x} + B,$$

where B is another constant. We therefore get

$$\frac{1}{y} = \frac{P}{x} \frac{A \int_1^x e^{-Qx} \frac{dx}{x} + B}{\frac{A}{x} e^{-Qx}} = P e^{Qx} \int_1^x e^{-Qx} \frac{dx}{x} + e^{Q(x-1)},$$

since $y=1$ when $x=1$

Write

$$-Ei(-x) = \int_x^\infty e^{-x} \frac{dx}{x} \quad 0 < x \quad (\text{exponential integral})$$

Then
$$\frac{1}{y} = P e^{Qx} [Ei(-Qx) - Ei(-Q)] + e^{Q(x-1)}$$

A further integration gives the ionic yield

$$I = p_1 RMQ \int_1^\infty \frac{e^{-Qx} dx}{P [Ei(-Qx) - Ei(-Q)] + e^{-Q}}$$

Make a change of variable

$$z = e^{-Q(x-1)},$$

write

$$s = e^{-Q},$$

and

$$Li(x) = \int_0^x \frac{dx}{\log x} \quad 0 \leq x < 1 \quad (\text{logarithmic integral}),$$

so that

$$Ei(-x) = Li(e^{-x}),$$

and

$$Li(x) = Ei(-\log 1/x),$$

then
$$I = p_1 RM \int_0^1 \frac{dz}{1 + \frac{P}{s} [Li(sz) - Li(s)]}$$

The exponential integral is a tabulated function and the last substitutions were designed to get the ionic yield in a form so that the final integration can be carried out most conveniently. The integration is taken between definite limits 0 and 1, and the integrand is a monotonic function increasing to 1 at $z=1$.

The relation between the ionic yield and the concentration of solute is seen to contain three parameters, P and constants multiplying I and C respectively, and their values must either be known or chosen to give the best fit with the experimental results. In the absence of any reliable data for the constants which are combined to form the parameters, we have to adopt the latter alternative.

The full line in Fig. 1 has been calculated using the following values for the parameters

$$P = 2.0, \quad p_1 RM = 6.0, \quad \frac{1}{M} \frac{Zpb^2}{4D} = 2.24 \text{ ml/g}$$

The choice of P is fairly critical, values of 1 or 5 giving definitely worse results. It is of interest to compare this value with that calculated from

$$P = \frac{\alpha N_0}{8\pi D},$$

taking $\alpha = 4 \times 10^{-10} \text{ cm}^3 \text{ sec}^{-1}$

and $D = 4.5 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$,

the mean of the H and OH diffusion constants (see Lea, 1947). We can assume 10^6 ion pairs per cm of electron track and therefore the number of radical pairs per cm N_0 , is $R \times 10^6$. The value of P is then 2.1 if we take six effective radicals per ion pair, or 0.35 if we take one. Since the constants can only be estimated approximately the agreement is satisfactory, and the shape of the experimental curve is therefore consistent with the recombination hypothesis.

The high ionic yield can be accounted for equally well by assuming either about six effective radicals (three pairs) per ion pair, or a chain reaction with an effective chain length of six, or by a combination of the two. This is on the assumption that p_1 is 1,

smaller values of p_1 would require larger value of R or M . One can only try to estimate the value of b , the mutual radius of the column of radicals, by analogy with the results of ion recombination in gases. From these we would infer that we should use a value of about 2×10^{-7} cm, the value is low because we are using a columnar theory, whereas the ions (and probably also the radicals) are formed in clusters along an electron track (see Kara-Michailova & Lea, 1940). Using this value for b and $Z = 4 \times 10^{-10}$ cm³sec⁻¹, we have to assume $p = 0.003$ for the non chain reaction, or $p = 0.019$ for the chain reaction, both cases therefore giving a low affinity of the radicals for the solute.

SUMMARY

1 Experiments are described showing the deamination by X rays of some amino acids and their derivatives, polypeptides and related nitrogenous

compounds. The relationship between the ionic yield and radiation dose has been studied in detail for glycine. The ionic yield rises from 0.3 to 2.9 for very dilute to nearly saturated solutions. The ionic yield for dry glycine was 2.96.

2 An explanation of the high ionic yield with glycine is attempted in terms of the radical hypothesis assuming either a chain reaction or the formation of radicals by excitation in addition to charge neutralization. The continuous rise of the yield is interpreted as being caused by recombination of radicals competing with deamination even at the highest concentrations of glycine, due to a low affinity of glycine to radicals. The explanation offered, although consistent with the experiments described, is not wholly satisfactory in view of the radiation effects on thiourea.

3 The biological significance of the results is discussed.

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An Investigation of the Intracellular Fluid of Calf Embryo Muscle

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(Received 2 February 1949)

Van Slyke (1913) estimated the amount of amino nitrogen extractable with boiling water from the skeletal muscle of the dog. Very few attempts have, however, been made to estimate or even to identify the particular amino acids present in the intracellular fluid of any tissue. The work on muscle has been summarized by Ussing (1943), who mentions estimations of arginine, histidine, cystine, glutamic acid, tyrosine and leucine plus valine. Since then Christensen, Streicher & Elbinger (1948) have

estimated glycine and glutamine in guinea pig muscle.

The present investigation concerns the substances in calf embryo muscle with molecules small enough to pass through a dialysis membrane; these mixtures are also compared with similar ones obtained from adult ox muscle and serum. Embryo muscle was used because extracts of this material are employed to maintain the growth of tissue cultures, although the stimulating effect of embryo extracts may be due

only to the large molecules present (Fischer, Astrup, Ehrensward & Øhlenschläger, 1948) it seemed of interest to try to ascertain whether there is any substance of low molecular weight detectable in embryo muscle but absent from that of the adult. The method used, that of partition chromatography on paper, while not suitable for quantitative estimations (with certain exceptions to be mentioned below), is particularly useful for an extensive survey.

EXPERIMENTAL

Investigations of intracellular fluids are made difficult by the fact that interference with the cell must undoubtedly lead to changes in its contents. Also the method used for the separation of the smaller from the larger molecules may have considerable effect on the results finally obtained. In the present work the tissue was minced and subjected to dialysis in the cold as described by Astrup, Ehrensward, Fischer & Øhlenschläger (1947). This technique was chosen because it seemed less likely to cause chemical change than either denaturation of the proteins by boiling (Fuller, Neuberger & Webster, 1947) or extraction with trichloroacetic or picric acid (Christensen *et al.* 1948). It has the advantage over extraction with ethanol (Awapara, 1948) that there is no danger of the amino acids being rendered insoluble or converted into esters. The chief disadvantage of this technique is that some enzymic breakdown of the large molecules is bound to take place during the dialysis. This is probably rather small in the case of the muscle proteins as it has been shown by Greenstein & Leuthardt (1946) that this tissue is low in proteolytic enzymes. However, as it would clearly be undesirable to carry on dialysis for too long a period, complete separation of the small molecules is impracticable.

When the solution obtained in this way was subjected to two dimensional chromatography two spots which could not at first be recognized were obtained in that part of the chromatogram characteristic of acidic compounds. Therefore the original solution was fractionated on a column of Amberlite IR 4, both the substances responsible for the unknown spots were found to be retained and were thus separated from the neutral and basic amino acids. Further investigation showed that the stronger of the two spots was due to aminoethylphosphate, a substance which had not previously been recognized as occurring in embryo muscle (Outhouse, 1937). The properties of the substance responsible for the other spot corresponded with those to be expected for a phosphoric ester of hydroxylysine. While the structure of this amino acid has not yet been fully clarified (Van Slyke, Hiller, MacFadyen, Hastings & Klemperer, 1940) it is of interest that aminoethanol could be obtained from either of the

suggested structures by fission at the linkage between the γ and δ carbon atoms. For convenience the hydroxylysine containing compound of the muscle extract will subsequently be referred to as hydroxylysinephosphate. As hydroxylysine has not previously been reported in combined form this substance has been further investigated. A preliminary report of its occurrence has already appeared (Gordon, 1948).

An attempt at a quantitative estimation of aminoethylphosphate and hydroxylysinephosphate has also been made by taking advantage of the ease with which these substances can be separated from other phosphorus containing compounds and from one another on one dimensional paper chromatograms. These analyses were performed on the fractions obtained from the Amberlite chromatogram, thus obviating the need to subtract the inorganic phosphorus which remained absorbed on the Amberlite until the final treatment with acid. Three fractions containing aminoethylphosphate and hydroxylysinephosphate were estimated separately so as to get information on the order of elution of these substances from the Amberlite. Each paper strip was cut up and eluted by the technique of Consden, Gordon & Martin (1947), guide chromatograms being used for the location of the spots. It would be simpler to treat the whole chromatogram with ninhydrin, but in the present work guides were used as a convenient means of keeping down the amounts of extraneous phosphorus in the eluates.

Preparation of material. As much as possible of the skeletal muscle was removed from a calf embryo weighing approximately 4 kg. After mincing, this weighed 622 g. An equal volume of water was added and the mixture was placed inside a cellophan tube and was allowed to dialyse with rocking at 5° against 2.4 l. of water for 20 hr. The solution outside the membrane was then removed (solution 1), replaced by a further 2.4 l. of water, and the dialysis repeated (solution 2). After solutions 1 and 2 had been concentrated *in vacuo* below 40°, estimations of N by micro Kjeldahl, and of total and inorganic P by the method of Kuttner & Lichtenstein (1932) were made. The results are shown in Table 1. Solutions 1 and 2 were then combined and will be referred to as the embryo muscle dialysate.

Table 1. Nitrogen and phosphorus obtained by dialysis of embryo mince

	Nitrogen (mg N)	Phosphorus (mg P)	
		Inorganic	Organic
Solution 1	393	100	88.0
Solution 2	214	72.5	34.3
Total	607	172.5	122.3

Two-dimensional chromatography of embryo muscle dialysate. Most of the inorganic salts present were removed from part of the above solution by the electro-dialytic method of Consden *et al.* (1947). An amount of the solution thus obtained

containing 0.52 mg N was subjected to two dimensional chromatography using phenol for 17 hr followed by *s*-collidine for 42 hr. The experimental conditions were the same as those described by Consden, Gordon & Martin (1944), except that 0.003% (w/v) of 8 hydroxyquinoline was added to the phenol, and coal gas was omitted. Whatman no. 4 filter paper was always used. A solution of 0.3% (w/v) NH_3 in the aqueous phase was employed at the bottom of the chamber for all the chromatography in phenol described in the present paper, except where an atmosphere containing acetic acid was used. Previous trials, in which the order of the solvents was reversed, gave much inferior results, confirming the observations of Dent (1947) with mixtures of this kind. The resulting chromatogram is shown in Fig. 1.

As a check on the identification of certain spots some of the muscle dialysate was made 6N with HCl and refluxed for 21 hr. After removal of the HCl *in vacuo* a similar two dimensional chromatogram was prepared.

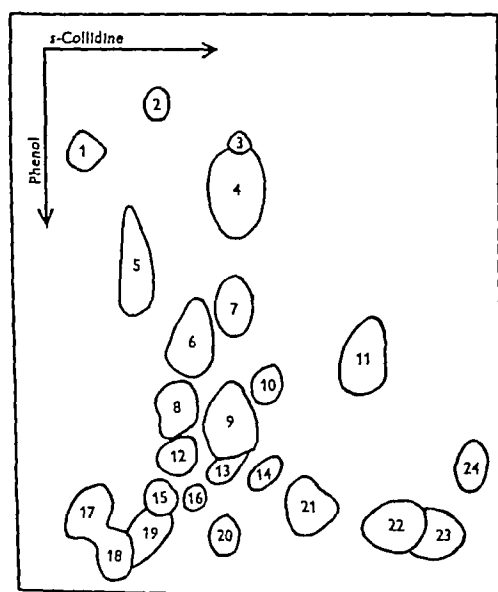


Fig. 1 Two dimensional chromatogram of calf embryo muscle dialysate. Developed for 17 hr in phenol followed by 42 hr in *s*-collidine. For explanation of the spots see Table 2.

Preparation and chromatography of adult ox muscle dialysate. A specimen of muscle from a fully grown animal was minced and extracted as described above for the embryo muscle. After removal of salt, a similar two dimensional chromatogram was prepared from an amount of solution containing 0.94 mg N. This rather large amount of material was used in order to show up any traces of hydroxylysine phosphate which might have been present.

A spot which appeared in the position expected for glutathione was easily separable from all other spots on a one dimensional chromatogram using phenol and hence it could conveniently be investigated by the elution technique of Consden *et al.* (1947). The eluate from a chromatogram developed in this way was hydrolysed, brominated and reinvestigated on a second one-dimensional chromatogram using phenol.

Preparation and chromatography of adult ox serum dialysate. The blood of an adult animal was defibrinated by stirring and the erythrocytes were removed by centrifugation. The serum thus obtained was dialysed as described above. After removal of salt from the material which had passed through the membrane, a similar two dimensional chromatogram was prepared using 0.53 mg N. The spots observed on the various two dimensional chromatograms are recorded in Table 2.

Table 2 Strengths of spots observed on two-dimensional chromatograms of calf embryo muscle dialysate (0.52 mg N), adult ox muscle dialysate (0.94 mg N) and adult ox serum dialysate (0.53 mg N).

(As the various amino acids give different amounts of colour with ninhydrin, uncorrected comparisons are only valid for the same amino acid. For the minimum amounts of each amino acid visible by means of ninhydrin on paper see Pratt & Auclair (1948). The numbers in brackets after each amino acid spot observed on the embryo muscle chromatogram refer to the spot numbers in Fig. 1.)

Strength of spot	Embryo muscle	Adult muscle	Adult serum
Very strong	Alanine (9) Glutamic acid (4) Glycine (6)	Alanine Glutamic acid Taurine	Alanine Glycine Leucine + iso leucine
Strong	Aminoethylphosphate (5) Glutamine (8) Taurine (11)	Carnosine Glutamine Glutathione Glycine Leucine + iso leucine	Glutamic acid Lysine Valine
Medium	β Alanine (12) Aspartic acid (3) Carnosine (19) Leucine + iso leucine (22)	Aspartic acid Lysine Serine Valine	Aspartic acid Arginine Glutamine Proline Serine
Weak	Arginine (18) Lysine (17) Phenylalanine (23) Hydroxylysine phosphate (1) Proline (20) Serine (7) Tyrosine (24) Valine (21)	β Alanine Arginine Methionine sulphoxide Aminoethyl phosphate Proline Tyrosine	γ Amino butyric acid Methionine sulphoxide Phenylalanine Taurine Threonine Tryptophan Tyrosine
Very weak	α Aminobutyric acid (14) γ Aminobutyric acid (15) Glutathione (2) Hydroxyproline (13) Methionine sulphoxide (16) Threonine (10)	Phenylalanine	α Aminobutyric acid Histidine

Chromatography of embryo muscle dialysate on Amberlite IR4. The method employed was the same as that of Consden, Gordon & Martin (1948) except for certain simplifications. Thus the Amberlite was used in the form obtained from the manufacturers without preliminary grinding and sieving. After alternate treatments with dilute HCl and NH_3 the

excess acid was removed from the chromatogram by elution with distilled water. Using a column 32 × 3.7 cm this required about 5 days, the column being ready for use when the eluate became 0.0013N (ascertained by titration).

Embryo muscle dialysate (50 ml) containing 404 mg N was applied to the chromatogram at a rate of not more than 150 ml/hr. After it had run in, it was followed by water at the same rate. Fractions were collected as shown in Table 3, the last fraction being eluted with 11 of N HCl. Each fraction was concentrated *in vacuo* and estimations made of N and of inorganic and total P. One dimensional chromatograms using phenol were also developed from samples of each fraction, salt having been removed electrolytically from fraction 1. The results shown in Table 3 indicate that aminoethylphosphate and hydroxylysinephosphate, under these conditions, are somewhat absorbed on Amberlite, although not so much as aspartic and glutamic acids. In a preliminary experiment it was found that a similar chromatogram will separate hypoxanthine from most of the neutral amino acids. The large white precipitate obtained on concentrating fraction 2 of the present experiment probably consisted mainly of this substance.

Further investigation of fractions from the Amberlite chromatogram

Phosphorus estimations on eluates from paper chromatograms. 20 µl of each of fractions 2, 3 and 4 containing respectively 48.5, 28.4 and 20.8 µg P were developed on one dimensional paper chromatograms using phenol for 17 hr. After drying, the chromatograms were cut up and eluted as described by Consden *et al.* (1947) except that about 1 ml of water was

allowed to run through each cut directly into a tube suitable for incineration. The P estimations were done as described by Kuttner & Lichtenstein (1932), except that half quantities of each reagent were used. The results are shown in Table 4. In order to indicate the limitations of the method the small amounts of P which originate from the paper have not been subtracted from the figures in columns 3, 6 and 9 of Table 4. Control experiments showed that an average of 0.05 µg P could be eluted from a 1 × 10 cm piece of Whatman no. 4 filter paper, down which nothing but the solvent has passed. If this correction is made, recoveries of 85, 101 and 93% are obtained by summing the P eluted from the whole of each chromatogram. In a control experiment on a sample of aminoethylphosphate (kindly supplied by Dr C. E. Dent) applied to a similar chromatogram, a recovery of 97% was obtained, using the same corrections.

Qualitative investigations of aminoethylphosphate and hydroxylysinephosphate

The materials used were eluted from one-dimensional chromatograms similar to those used for the quantitative investigation described above. In order to obtain a larger amount, 0.3 ml of fraction 3 was applied to a sheet of paper 48 cm wide. Only the cuts which were expected to contain aminoethylphosphate or hydroxylysinephosphate were eluted. The solution of the latter obtained in this way was further purified by a similar chromatographic procedure in which *n*-butanol containing 20% of acetic acid was used instead of phenol. In a second experiment *s*-collidine was substituted for *n*-butanol 20% acetic acid, without noticeable affecting the product.

Table 3 Fractions obtained from embryo muscle dialysate by chromatography on Amberlite IR 4

Volume of eluate (ml.)	Fraction					Total
	1	2	3	4	5	
Water	500	240	1640	360	—	2740
N HCl	—	—	—	—	1000	1000
N as % of total N	73.8	6.9	2.8	1.0	8.7	93.2
Inorganic P as % of total inorganic P	—	—	—	—	91.4	91.4
Organic P as % of total organic P	7.0	1.4	11.4	1.4	72.7	93.9
Substances identified by chromatography on paper						
Hydroxylysinephosphate	—	V weak	Medium	Weak	—	—
Aspartic acid	—	—	—	—	Weak	—
Glutamic acid	V weak	V weak	Strong	V strong	V strong	—
Aminoethylphosphate	—	V weak	Medium	Weak	—	—
Neutral and basic amino acids	V strong	V weak	V weak	—	—	—

Table 4 Amounts of phosphorus eluted from cuts from paper chromatograms

Cut no	Fraction 2			Fraction 3			Fraction 4		
	Breadth (cm)	P (µg)	P as % of total organic P	Breadth (cm)	P (µg)	P as % of total organic P	Breadth (cm)	P (µg)	P as % of total organic P
1	4.1	1.8	0.1	3.7	1.9	0.7	3.8	1.8	0.1
2	3.6	1.8	0.1	2.8	1.0	0.3	2.6	0.7	0.0
3	4.4	13.1	0.4	4.9	6.0	2.3	4.6	4.4	0.3
4	4.5	0.8	0.0	3.1	0.5	0.1	2.9	0.3	0.0
5	8.8	12.9	0.4	5.8	13.0	5.1	7.5	10.7	0.7
6	8.0	0.0	0.0	8.0	0.4	0.0	8.2	0.6	0.0
7	9.0	0.4	0.0	8.3	0.6	0.1	8.0	0.3	0.0
8	9.0	13.4	0.4	8.3	7.5	2.8	8.0	2.8	0.2
Total	51.4	44.2	1.4	44.9	30.9	11.4	45.6	21.6	1.3

Enzymic hydrolyses The enzyme used was the phosphatase of dog faeces, purified according to Armstrong (1935), stages A and B, and subsequently dialysed. It was allowed to act for 3 hr at 40° on each of the above eluates (approximately 10 µg of enzyme/µg P). The pH of each solution was adjusted to 8.8 with NH_3 . After drying *in vacuo* the products were investigated on one dimensional chromatograms using phenol, phenol + 0.003% 8 hydroxyquinoline in an acidic atmosphere (5% acetic acid at the bottom of the chamber) and ϵ collidine as solvents. The R_F values of the spots due to the original substances and those due to their hydrolysis products are shown in Table 5.

Table 5 R_F values of certain phosphorus containing substances of calf embryo muscle

Solvent Addition	Phenol 0.003% 8 hydroxy quinoline + 0.3% NH_3	Phenol 0.003% 8 hydroxy quinoline + 5% acetic acid	ϵ Collidine
Hydroxylysine phosphate	0.12	0.17	0.0
Hydroxylysine	0.66	0.22	0.14
Aminoethyl phosphate	0.31	0.39	0.05
Aminoethanol	Front	0.67	0.22

The nature of the amino acid set free from hydroxylysine phosphate was further checked by admixture with a specimen of hydroxylysine kindly made available by Dr J G Heathcote. No separation could be obtained using any of the above solvents. The identity of the amino ethylphosphate was similarly checked both before and after hydrolysis with the enzyme, against a specimen kindly supplied by Dr C E Dent. Again no separation was obtained. As can be seen from Table 5 the difference between the R_F values of hydroxylysine and hydroxylysinephosphate in phenol 5% acetic acid is too small for complete separation to be easily possible. The results of such chromatography of samples of hydroxylysinephosphate after treatment with the phosphatase were often difficult to interpret since more than one spot could usually be observed which had moved more slowly than hydroxylysine and was incompletely separated from it. However, when a sample of hydroxylysinephosphate, treated with the phosphatase under the conditions described above, was developed on a two dimensional chromatogram using phenol 5% acetic acid for 17 hr followed by phenol 0.03% NH_3 for 17 hr, only one spot was observed. This had the expected R_F values for hydroxylysine.

Acid hydrolysis Small amounts of the aminoethylphosphate and hydroxylysinephosphate were hydrolysed at 100° for 24 hr in 8N HCl as described by Consden *et al* (1947). After removal of HCl, chromatography of the hydrolysate of aminoethylphosphate showed only spots corresponding to unchanged material and aminoethanol. From hydroxylysinephosphate were formed only spots corresponding to unchanged material and hydroxylysine and in phenol acetic acid a faint spot (R_F 0.07) of unknown origin.

RESULTS AND DISCUSSION

Although the full characterization of the substance referred to in the present paper as hydroxylysine phosphate must await its isolation, perhaps from

a tissue in which it occurs in higher concentration, its presence in embryo and its absence from adult muscle remains the most interesting finding of the present work. How little hydroxylysinephosphate can be present in the adult muscle is indicated by the failure to obtain a spot due to this substance even though considerably more than the usual amount of the dialysate was used for this chromatogram. It is also of interest that while a spot due to aminoethylphosphate was formed from both the muscle extracts, that from the embryo was much the strongest. Neither of these substances was detectable in the serum of an adult animal. Rather strong spots due to aminoethylphosphate, but none due to hydroxylysinephosphate, were observed on chromatograms of similar dialysates made from Rous sarcoma (Mill Hill) and from Ehrlich mouse sarcoma. (The dialysis and chromatography of these two sarcomas was done by Ing R Stjernholm to whom thanks are due.)

The glutathione spot formed from the embryo muscle was very weak in contrast to that formed from the adult muscle. When the latter was separated and hydrolysed as described above, the hydrolysate on further chromatography yielded only spots due to glutamic acid, glycine and cysteic acid (from cystine). Thus the occurrence of serinephosphate, which has almost identical R_F values with glutathione in both solvents, was excluded. Not surprisingly, carnosine was absent from serum, but present in considerable amounts in both the tissue dialysates. Strong spots due to taurine were formed from both the tissue dialysates, while a weak spot appeared on the serum chromatogram. A spot due to this substance has been observed by Dent (1948) on chromatograms of human plasma.

Turning now to the α amino acids, it is noticeable that the concentration of leucine and/or isoleucine, valine and lysine in the embryo muscle dialysate is relatively low compared with the concentration of these amino acids in serum. An intermediate amount seemed to be present in the adult muscle dialysate. On the other hand, glutamic acid and glutamine are rather stronger in the tissue dialysates than in the serum.

Very weak spots were detected on the chromatograms of embryo muscle extract and adult serum in the positions to be expected for α and γ amino butyric acids. Also a weak spot, probably due to methionine sulfoxide, was observed on all three two-dimensional chromatograms. Dent (1948) has observed similar spots on chromatograms of various natural mixtures, and has checked their identity by the addition of known samples of each substance. However, until these substances have been isolated and fully characterized their recognition must remain somewhat tentative. In the present work such checks were not made. Confirmation was

obtained of the stability of the various substances towards acid by chromatographic examination of a sample of the embryo dialysate which had been refluxed for 17 hr with 6N HCl. Only the spots due to glutathione, glutamine and carnosine had disappeared.

Needham (1931) has summarized many investigations of the concentration of amino N in the blood of human and rabbit embryos. The amounts present were always rather greater than in the corresponding maternal blood. Although this work does not seem to have been extended to the tissue fluids of the embryo it is likely that these also must contain higher concentrations of amino acids than do the maternal tissues. Even if such differences are shown to be the case it seems likely that they will be much smaller than the corresponding differences of concentration of hydroxylysinephosphate and aminoethylphosphate noticed in the present investigation between embryo and adult or muscle. The only quantitative estimations of these substances so far attempted have been carried out on the fractions obtained from the embryo muscle extract after chromatography on Amberlite IR 4. If the amounts of P believed to be due to hydroxylysinephosphate in the various fractions are summed, it appears that this compound represents 3% of the organic or 1% of the total P of the embryo muscle dialysate. Similarly, aminoethylphosphate represents 6% of the organic or 2% of the total P. If unknown P containing substances have moved at similar rates on the chromatograms used, these figures will of course be too high. That any correction would be small, however, is suggested by an experiment in which hydroxylysinephosphate, after elution from a phenol chromatogram, was rechromatographed using *n* butanol 20% acetic acid, on elution of appropriate sections of the second chromatogram no separation of P from the hydroxylysinephosphate was found.

In order to calculate the total quantities of these substances present in the muscle of the original embryo, account must be taken of the fact that the dialysis was not taken to completion. An indication of the proportion of the low molecular weight substances actually obtained can be gained from Table 1 which gives the amounts of N and P passing through the membrane after the successive periods of dialysis. Thus, if it is assumed that the same proportion of the N would have passed through the membrane if the

solution had been subjected to further successive dialyses, it appears that some 70% of the non-protein N was removed from the mince. On a similar assumption 67% of the available P was obtained. The considerably higher proportion of inorganic P obtained during the second period of dialysis is an indication of the rapid breakdown of organic phosphates which was taking place.

One of the objects of the present work was to test the effect on tissue cultures of any substance found to occur only in embryo muscle. This has not yet been attempted with the fractions containing hydroxylysinephosphate, but as aminoethylphosphate seemed to be so much more concentrated in the embryo muscle dialysate than in the adult, it was subjected to test. The system used was the usual one of Fischer & Astrup (1942) employing chick heart fibroblasts grown in a Carrel flask. Using the synthetic aminoethylphosphate + cystine, glycine and glutamine, excellent growth was obtained. Further experiments would be necessary to decide whether aminoethylphosphate is more than simply a good source of P for tissue cultures.

SUMMARY

1 The amino acids and peptides of the intracellular fluids of calf embryo muscle have been examined by two-dimensional chromatography on paper and compared with similar mixtures from muscle and serum of the adult animal.

2 After removal of basic and neutral substances by chromatography on Amberlite IR 4 the acidic components were fractionated by one dimensional paper chromatography. Sections corresponding to the spots observed on treatment with ninhydrin were eluted and the eluates were analysed for phosphorus and further examined.

3 Hydrolysis and repeated chromatography of these eluates indicated that aminoethylphosphate and hydroxylysine in a form combined with phosphorus are present. The former substance was found to represent 2% and the latter 1% of the dialysable phosphorus of the minced muscle.

I wish to thank Dr Albert Fischer for suggesting this problem and also for hospitality in his laboratory. This work was supported by the Donner Foundation to whom thanks are due.

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The Effect of Dietary Oleic and Palmitic Acids on the Composition and Turnover Rates of Liver Phospholipins

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(Received 2 February 1949)

Ozaki (1927) found that rats fed on diets containing 20% oleic acid gained weight, whereas rats on diets containing similar amounts of palmitic or stearic acids lost weight. These weight changes indicate differences in the metabolism of oleic acid as compared with that of the saturated fatty acids. Channon & Wilkinson (1936) have shown that on low-protein, high fat diets, lipid accumulation in the liver of the rat varies directly with the proportion of saturated fatty acids in the dietary fat, their fatty liver producing diets were deficient in choline and accumulation of fat was prevented by choline. It was suggested that choline was involved in the desaturation of fatty acids. The differences in accumulation of fat may have been due to different phospholipin turnover rates determined by the varying supply of saturated fatty acids.

In the experiments reported in this paper low-protein diets containing pure palmitic and oleic acids were fed to rats in such a way as to eliminate effects due to different food intakes or absorption coefficients of the fatty acids. An attempt was made to correlate the different lipid accumulations with differences in liver phospholipin turnover rates.

METHODS

Male albino rats of the Wistar strain were used. All animals were bred by sister brother matings from a single pair. The starting weight chosen was 150 g, as younger animals showed excessive weight loss, and some died on the palmitic acid diet described in Table 1. The complication of an additional requirement for lipotropic factors for growth was also more likely to be avoided at this weight.

In a preliminary experiment to determine the percentage absorption of pure oleic and palmitic acids when fed at a level of 27%, it was found that 70% of the ingested palmitic

acid, and 90% of the oleic acid were absorbed. The proportions of fatty acid and cellulose powder in the two diets were therefore adjusted so that for both groups of animals the amounts of fat absorbed and the values of the diets as sources of energy were the same.

Table 1 Composition of fatty acid diets

	Oleic acid diet	Palmitic acid diet
Casein (Labco) (%)*	8	8
Fatty acid (%)	21	27
Glycerol (%)	3	3
Sucrose (%)	50	50
Yeast (%)	5	5
Salts (%)†	5	5
Cellulose powder (%)‡	8	2

* When choline supplements were given the casein was sprayed with a solution of choline chloride to give 30 mg choline/10 g diet.

† NaCl 22 g, CaHPO₄ H₂O, 130 g, potassium citrate, 125 g, MgSO₄ 7H₂O 30 g, ferric citrate, 5 g, 0.7 g of trace mixture (KI, 12 g, NaF, 10 g, MnSO₄ 2 g, Cu₂I₂, 1 g, KAl(SO₄)₂, 1 g, ZnSO₄ 7H₂O, 1 g, de Loureiro, 1931).

‡ Grade A '100 mesh' 'solka flocc', a highly purified cellulose powder obtainable from Johnsen, Jorgensen and Wettre Ltd, London.

Animals were starved for 24 hr and then placed on the diets for 12 days. The palmitic acid diet was not eaten so readily as the oleic acid diet and therefore approximate pair feeding between litter mates was adopted. Controls were fed *ad lib* on a stock diet consisting of skimmed milk powder, 20%, ground yellow maize, 18%, rolled oats 15%, barley meal, 15%, biscuit meal, 15%, wheat germ, 5%, palm kernel oil, 5%, dried yeast, 5% and bran, 2%. Each animal received 10 μ Ci of ³²P as Na₂H³²PO₄ in distilled water by intraperitoneal injection, and was killed by decapitation 4 hr later. In order to reduce the effect of recently ingested fat on the specific activities of liver phospholipins, food and water were removed from the animals 4 hr before the injection of ³²P. (Specific activity (SA) = % injected ³²P/mg phosphorus).

Livers were homogenized with anhydrous Na_2SO_4 and extracted twice with ethanol and three times with 2:1 ethanol ether in a small blender and re extracted with light petroleum. Phospholipins were precipitated with MgCl_2 and acetone. Amounts of phospholipins were estimated by multiplication of the amounts of phosphorus by 22.7 (Artom & Fishman, 1943a). Inorganic phosphate was extracted from the livers by the method of Kaplan & Greenberg (1944a, b). Choline containing phospholipins were separated by the method of Taurog, Entenman, Fries & Chaikoff (1944) and phosphorus was estimated by reduction of phosphomolybdate with sulphite quinol. The percentage choline containing phospholipin was obtained from the ratio of eluted to total phosphorus. Iodine numbers were estimated by Yasuda's (1931) method and cholesterol and cholesterol esters by the method of Popják (1943).

Radioactivities were determined with a G.T. 11 type G.M. tube arranged as an immersion counter, with Neher Harper extinction and feeding into a Type 200 scaling unit.

All analyses were carried out in duplicate.

RESULTS

Weight loss and lipid accumulation

Animals pair fed on the oleic and palmitic acid diets lost weight in both groups. The animals fed on oleic acid lost weight in inverse proportion to the amount of food consumed ($r = -0.791$, $n^2 = 16$, $p = 0.0001$,

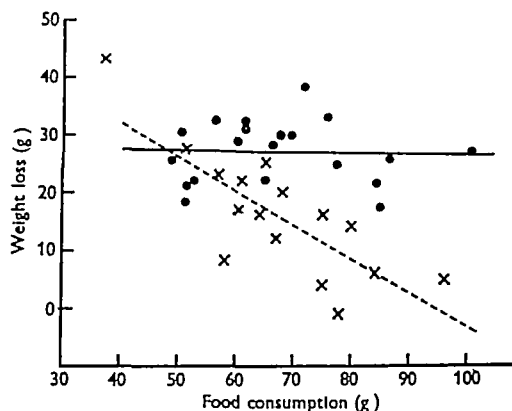


Fig 1 Relation between total food consumption and loss of body weight for rats fed diets containing oleic and palmitic acids. The lines of regression have significantly different regression coefficients, z (palmitic) $= -0.012$, z (oleic) $= -1.074$, $n^2 = 36$, $p = 0.005$, \bullet = palmitic acid fed animals, \times = oleic acid fed animals.

all statistical symbols are those used by Fisher, 1944), whereas the weight loss in the animals fed palmitic acid was not related to food consumption ($r = -0.012$, $n^2 = 20$). All animals fed palmitic acid had similar weight losses, irrespective of the amount of liver lipid accumulation. Litter mates, fed the diets with and without choline supplementation, lost the same weight.

The animals fed palmitic acid drank on an average 25% less water than those fed oleic acid, but excreted less urine. The difference between water intake and urinary excretion was the same in both groups, and therefore could not have contributed to the weight loss.

Analyses of water and fat contents of eviscerated carcasses and skins were done on six animals from each dietary group. It can be seen from Fig 2 that the palmitic acid-fed animals lost approximately 10 g of fat over the 12 day feeding period, independently of the food eaten, whereas the oleic acid fed animals lost from 0 to 10 g of fat, depending on the food consumption. The ratio of non-fat solids to water was the same for all groups.

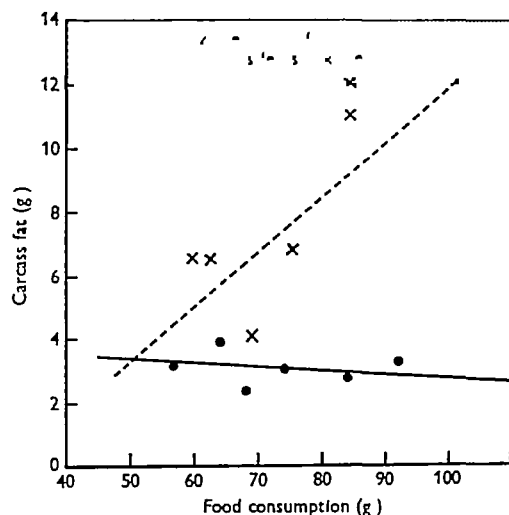


Fig 2 Relation between total food consumption and the fat content of eviscerated carcasses of rats fed diets containing oleic and palmitic acids. The lines of regression have significantly different regression coefficients, z (palmitic) $= -0.295$, z (oleic) $= +1.83$, $n^2 = 12$, $p = 0.01$, \bullet = palmitic acid fed animals, \times = oleic acid fed animals.

Table 2 shows that in the earlier experiments, a large and remarkably constant accumulation of fat in the liver was obtained with palmitic acid feeding. In the oleic acid fed animals a smaller and more variable amount of fat accumulated. A sudden change occurred in the fifth generation of rats, there was no accumulation of fat in the livers of litter mates of the fifth generation fed diets containing oleic or palmitic acid with or without choline supplements. A single litter among the earlier rats had already shown signs of this tendency.

A choline supplement was only fed to animals of the fifth generation. There is, therefore, no evidence to show that choline would have cleared the lipid accumulations of the animals in the earlier experiments. However, feeding palmitic acid and choline did reduce the percentage of fat in the liver to a level

lower than that of control animals on the stock diet ($t=4.26$, $n^2=19$, $p=0.001$) This effect was not found in the animals fed oleic acid and choline

There was no correlation between the percentage of fat in the liver and weight loss or food consumption in any group

Average values of iodine numbers of liver triglyceride after 12 days feeding were 75 for palmitic acid fed animals (irrespective of the extent of lipid accumulation), 110 for oleic acid fed animals and 130 for control animals

Fat accumulation was accompanied by proportional increases in ester cholesterol, irrespective of the nature of the dietary fat A constant relationship between ester cholesterol and the deposition of liver triglyceride has been found by Best, Lucas, Patterson & Ridout (1946)

Table 2 *Litter variations in total liver lipids*

(Values expressed as % fresh weight)

	No of animals	Palmitic acid fed animals	No of animals	Oleic acid fed animals
Litters of third and fourth generation	8	20.70 S.E. = 0.590	10	8.72 S.E. = 0.910
Isolated litter of fourth generation	3	11.70 S.E. = 1.23	2	5.95 S.E. = 0.350
Litters of fifth generation	9	6.84 S.E. = 0.530	2	6.20 S.E. = 0.360

S.E. = standard error of mean

Estimation of rate of turnover of phospholipin phosphorus

The ratio, specific activity of phospholipin phosphorus/specific activity of inorganic phosphate at 4 hr which is a function of the percentage rate of turnover of the phospholipin molecules, is referred to subsequently as 'relative specific activity' (Hevesy, 1938) The total amount of any metabolite turned over in a given time depends on the number of molecules present and their percentage turnover rate As a measure of this total turnover rate, the product of the amount of metabolite and its relative specific activity may be employed and is referred to in this paper as 'total relative activity'

A preliminary experiment was done to assess the true percentage molecular turnover rate of total and choline containing phospholipin phosphorus in rats of the weight and strain used in this experiment In attempting to estimate the true rate of renewal of phospholipin phosphorus, other workers have employed a method of constant intravenous perfusion with a solution of radioactive phosphate, with the intention of keeping the specific activity of the inorganic phosphate constant throughout the experiment Estimates of the turnover rate of phos-

pholipin phosphorus have been based on the relationship between the specific activity time curves of ^{32}P in phospholipins and that of ^{32}P in inorganic phosphate This method is inconvenient in practice and therefore a method using a single injection of ^{32}P was devised While the present work was in progress a similar procedure was described by Bollman, Flock & Berkson (1948) Male rats (150 g) were maintained for 8 days on a diet of 50 % sucrose, 22 % casein, 12 % lard, 5 % yeast, 5 % salt mixture, 5 % cellulose powder and adequate supplements of concentrated vitamins A and D Vitamin E was not given These animals received injections of $\text{Na}_2\text{H}^{32}\text{PO}_4$ and were killed at 0.5, 1, 2, 4, 6 and 8 hr intervals after injection Food and water were removed from all animals 4 hr prior to the injection of the first animal From the measured time curve of specific activity of liver inorganic phosphate, theoretical curves for specific activities of phospholipin phosphorus were obtained for different selected percentage molecular turnover rates by the following procedure The mean value of specific activity of inorganic phosphate over the first half hour period, $[\text{SA PO}_4]_{0-0.5 \text{ hr}}^{0.5 \text{ hr}}$, was measured from the phosphate activity curve The assumption was made that freshly synthesized phospholipin molecules containing ^{32}P are as available for degradation as the original non active phospholipin (Chalkoff, 1942) If the immediate precursor of phospholipin is taken to be in rapid equilibrium with either the inorganic phosphate or the phospholipin, an estimate of the specific activity of the latter at the end of the first half hour $[\text{SA PL}]_{0.5 \text{ hr}}$ can be obtained from the following equation

$$[\text{SA PL}]_{0.5 \text{ hr}} = \frac{\{[100 - \Delta\text{PL}] \times 0\} + \{\Delta\text{PL} \times [\text{SA PO}_4]_{0-0.5 \text{ hr}}^{0.5 \text{ hr}}\}}{100},$$

where ΔPL = the percentage of phospholipin phosphorus atoms renewed in 0.5 hr Then the specific activity at the end of the n th 0.5 hr

$$[\text{SA PL}]_n = \frac{\{[100 - \Delta\text{PL}] \times [\text{SA PL}]_{n-1}\} + \{\Delta\text{PL} \times [\text{SA PO}_4]_{n-1}^n\}}{100}$$

A theoretical curve of liver phospholipin specific activity for a selected percentage molecular turnover of 25 % in 4 hr was found to be the best fit to the practical curve, and is shown together with the latter in Fig. 3 The measured specific activity time curves for total and choline containing phospholipins were in close agreement

If phosphate enters and leaves the molecules at the same rate as the fatty acid components, then a renewal of 25 % of the phosphate in 4 hr means that 100 mg of phospholipin is concerned in the metabolism of 97.5 mg of fatty acid or 108 mg of

triglyceride/day Allowing for the normal variation in percentage molecular turnover rates, differences of 30 % in fatty acid metabolism by the phospholipins, i.e. about 30 mg /day, should be detectable

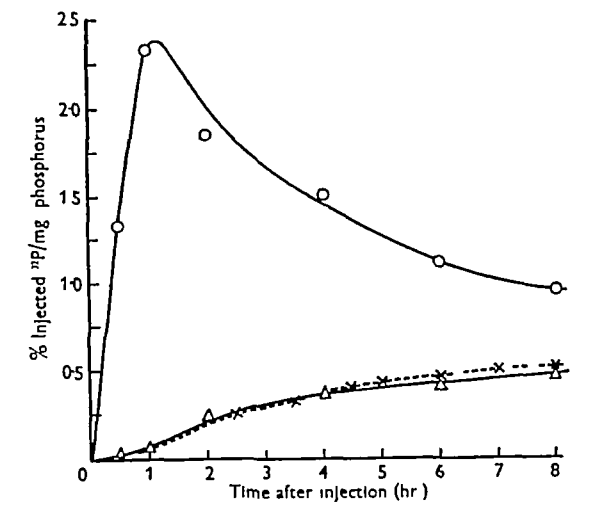


Fig 3 Specific activity time curve for liver inorganic phosphate and total phospholipin phosphorus, —○—=inorganic phosphate, —△—=total phospholipin phosphorus, ---x---=theoretical curve for a turnover of 25 % of phospholipin phosphorus in 4 hr The curve for choline containing phospholipin phosphorus is not shown as it closely approximated to the curve for total phospholipin

The effects of diet on phospholipin turnover rates

A significant correlation between the specific activity of liver inorganic phosphate and the amount of lipid/unit of dry defatted liver weight has been found ($r=0.309$, $n^1=42$, $p=0.05$) Table 3 shows that this relationship was independent of the type or amount of fat ingested Specific activities of the liver inorganic phosphate were not correlated with the final body weight, and therefore the differences in specific activity are unlikely to be related to changes in plasma volume

There were no significant differences in relative specific activities or total relative activities of the phospholipins on an absolute or body weight basis at

4 hr between any of the dietary groups with or without choline supplements (Table 4) There was less variability in the phospholipin relative specific activities of the animals on low protein, high fat diets than was shown by animals on the more mixed stock diet

The values of specific activities at 4 hr of choline containing phospholipins were on the whole close to those for total phospholipin in all groups of animals, suggesting that different fat accumulations were not related to differences in the percentage molecular turnover rates of the phosphorus of the choline and non choline containing phospholipin fractions This is confirmed by the close agreement of the specific activity time curves of the choline containing and total phospholipins

Changes in amounts and composition of phospholipin

From Table 4 it can be seen that the amount of liver phospholipin per unit of final body weight is significantly lower in the animals from the third and fourth generations fed palmitic acid for 12 days than in animals of the same generation fed oleic acid ($t=2.34$, $n^1=22$, $p=0.035$) or the control diet ($t=2.65$, $n^1=22$, $p=0.017$) There was no significant difference between the third and fourth generation oleic acid fed animals and the controls The amount of phospholipin per unit of body weight was not related to the liver lipid accumulation in third and fourth generation animals fed palmitic acid as these did not differ significantly from the fifth generation animals which failed to accumulate liver fat

Supplementation of the diet with choline did not alter this quantity either in palmitic or oleic acid fed animals of the fifth generation The ratio of dry defatted liver weight to body weight was the same in all groups Therefore, there were similar differences in amounts of phospholipin on a dry defatted liver weight basis

The composition of the liver lipids of those rats in which the phospholipins were separated into choline containing and non choline containing fractions is shown in Table 5

Table 3 Liver lipids and specific activity of liver inorganic phosphate (4 hr after injection of ³²P)

Description	No of animals	Final body wt (g)	Fat absorbed in 12 days (g)	Liver lipid (% dry defatted liver wt)	Specific activity liver inorganic phosphate
Third and fourth generation palmitic acid fed animals (with fatty livers)	10	115	12.6	98.4 S.E.=9.43	2.56 S.E.=0.179
Oleic acid fed animals	13	125	12.5	36.1 S.E.=3.98	2.27 S.E.=0.103
Fifth generation palmitic acid fed animals	9	114	11.8	30.2 S.E.=3.05	2.08 S.E.=0.113
Controls	10	140	—	27.4 S.E.=1.54	1.88 S.E.=0.191

Table 4 *Liver lipids and phospholipin radioactivities*
(4 hr after injection of ^{32}P)

Description	No of animals	Liver fat (% fresh wt)	Total liver phospholipin (g) $\times 10^4$ /body wt (g)	Relative specific activity $\times 100$	Total relative activity (g)	Total relative activity (g) $\times 100$ /body wt
Palmitic acid fed animals						
Third and fourth generation (with fatty livers)	11	18.24 S.E. = 1.37	8.38 S.E. = 0.093	29.98 S.E. = 2.04	2.95 S.E. = 0.275	2.55 S.E. = 0.222
Fifth generation	9	6.84 S.E. = 0.532	8.14 S.E. = 0.470	31.46 S.E. = 2.20	2.94 S.E. = 0.981	2.59 S.E. = 0.254
Fifth generation palmitic acid + choline fed animals	9	4.74 S.E. = 0.250	7.79 S.E. = 0.239	35.60 S.E. = 2.25	3.18 S.E. = 0.257	2.74 S.E. = 0.218
Oleic acid fed animals						
Third and fourth generation	11	8.26 S.E. = 0.814	10.50 S.E. = 0.270	29.70 S.E. = 1.76	4.15 S.E. = 0.639	3.27 S.E. = 0.422
Fifth generation	2	6.20 S.E. = 0.360	9.42 S.E. = 1.89	29.10 S.E. = 6.30	3.35 S.E. = 0.370	2.68 S.E. = 0.340
Fifth generation oleic acid + choline fed animals	5	5.30 S.E. = 0.500	9.51 S.E. = 1.03	31.50 S.E. = 1.92	3.62 S.E. = 0.621	3.00 S.E. = 0.414
Controls						
Third and fourth generation	11	6.06 S.E. = 0.190	13.37 S.E. = 1.86	38.90 S.E. = 6.00	3.77 S.E. = 0.363	2.73 S.E. = 0.269

Table 5 *Changes in phospholipin composition and liver lipid accumulation*

Description	No of animals	Liver fat (% fresh wt)	Acetone soluble lipins (mg)	Total phospholipin (mg)	Choline containing total phospholipin (%)	Amount choline containing phospholipin (mg)	Amount non choline containing phospholipin (mg)
Palmitic acid fed animals							
Third and fourth generation (with fatty livers)	6	19.7 S.E. = 1.59	881.8 S.E. = 120.8	105.5 S.E. = 5.17	59.1 S.E. = 4.18	63.03 S.E. = 6.43	42.87 S.E. = 3.98
Fifth generation	9	6.84 S.E. = 0.532	150.9 S.E. = 22.3	92.5 S.E. = 4.63	65.0 S.E. = 5.17	58.5 S.E. = 2.71	34.3 S.E. = 6.25
Fifth generation palmitic acid + choline fed animals	9	4.74 S.E. = 0.250	85.2 S.E. = 7.20	88.9 S.E. = 4.04	71.5 S.E. = 2.25	63.2 S.E. = 4.06	25.5 S.E. = 3.77
Oleic acid fed animals							
Third and fourth generation	7	9.57 S.E. = 1.17	334.1 S.E. = 68.4	140.1 S.E. = 21.1	47.9 S.E. = 4.01	66.4 S.E. = 9.74	73.6 S.E. = 13.9
Fifth generation	2	6.20 S.E. = 0.360	155.0 S.E. = 26.0	118.0 S.E. = 13.0	64.2 S.E. = 9.25	76.9 S.E. = 19.1	41.0 S.E. = 6.20
Fifth generation oleic acid + choline fed animals	5	5.30 S.E. = 0.500	117.4 S.E. = 19.9	114.3 S.E. = 16.2	82.0 S.E. = 1.98	93.6 S.E. = 13.1	20.6 S.E. = 4.06
Controls							
	5	6.18 S.E. = 1.13	175.1 S.E. = 45.7	202.0 S.E. = 29.1	57.9 S.E. = 7.47	109.6 S.E. = 9.28	92.4 S.E. = 29.6

In the oleic acid fed animals there is an indication that as the choline containing phospholipin increased there was a reduction in the amount of non choline containing phospholipin with resulting constancy in the total phospholipin. The palmitic acid fed animals, however, showed a remarkable constancy in amounts of choline containing phospholipin, so that reductions in total amounts of phospholipin were due to reductions in the non choline containing fractions. There is also some indication that reductions in amounts of non choline containing phospholipin in both oleic and palmitic acid fed

animals were accompanied by reductions in amounts of acetone soluble lipids.

DISCUSSION

Figs 1 and 2 show that in both oleic and palmitic acid fed animals depots are depleted due to a low food intake. The fact that an increased food consumption does not reduce the weight loss of the palmitic acid fed animals indicates the presence of some factor in addition to the amount of food eaten. Hodge, MacLachlan, Bloor, Stoneburg, Oleson &

Whitehead (1941) and MacLachlan (1944) have obtained evidence that in fasting mice the depot fat which is metabolized has an iodine number of 80. If palmitic acid provides a large proportion of the energy value of the food supplied to the body, and if in the rat an iodine number of the same order is obligatory for fat combustion by the liver and possibly elsewhere in the body, then it is likely that either the palmitic acid will not be metabolized and will accumulate, or the iodine number of the fat being metabolized will either be raised by desaturation or by blending with unsaturated fatty acids. The present work indicates that the latter is the case. Hodge *et al.* (1941) found that the liver of the fasting mouse metabolizes 92% of the depot fat on the first day, a maximum of 8% accumulates in the liver. Animals in our experiment consumed up to 20 g of palmitic acid, and depleted their depots of approximately 10 g of fat, the highest accumulation of fat in the liver was only 750 mg. The fact that the dietary fat had an iodine number of zero, but that the liver triglyceride maintained an iodine number of 75 indicates that a considerable proportion of the liver fat accumulation is not dietary palmitic acid. This may be partly due to desaturation, but the depletion of the depots indicates that mobilization and blending of the fatty acids of the depots with palmitic acid are also occurring. Release of lipids from the depots may explain the diminished water intake of the palmitic acid fed animals as being due to the operation of a depot mobilizing pituitary factor, influencing the water balance (Best & Campbell, 1936).

The significant reduction of percentage of liver fat below the normal level when choline is fed with palmitic acid may indicate that choline has a particularly important role in the metabolism of saturated fats. Work is in progress to determine if choline restores the iodine number of the liver triglyceride to normal levels.

Hodge *et al.* (1941) found a constant level of phospholipin in their fasting mice despite combustion of considerable amounts of depot fat by the liver, and interpreted this as indicating a relatively unimportant participation of the phospholipins in this combustion. Studies of the levels of liver phospholipins in fasting have been complemented by work with ^{32}P , by Hodge, MacLachlan, Bloor, Welch, Kornberg & Falkenheim (1947), who found that the specific activity of liver phospholipins showed a sharp increase on the second day of fasting. However, Kaplan & Greenberg (1944c) have shown that rats fasted for 3 days had marked increases in the specific activity of their liver inorganic phosphate, which could in turn produce a rise in the specific activity of the liver phospholipins without any real change in the turnover rate of the latter. A re-investigation of the turnover of liver phospholipin

during fasting including measurements of activities of inorganic phosphate would appear to be worth while, but at present it seems that evidence for increased turnover rates produced by release of depot fats to liver is ambiguous.

The correlation of specific activity of liver inorganic phosphate with amounts of liver lipid shows the importance of measuring the specific activity of liver inorganic phosphate or some other precursor in work on phospholipin turnover rates in which different lipid accumulations are to be expected.

The differences in specific activity of liver inorganic phosphate may be due to several factors. The accumulation of excess liver lipid may alter the permeability of liver cells to plasma inorganic phosphate. In this connexion it is of interest that Flock, Bollman & Mann (1936) have found a marked reduction in liver inorganic phosphate in dogs with fatty livers. Derangements of liver phosphate metabolism due to the presence of excess lipid are also indicated by the work of Ennor & Stocken (1948) who found increases in amounts of the labile phosphate fractions in fatty livers produced by carbon tetrachloride poisoning.

Our observations indicate a remarkable constancy of percentage molecular turnover rates even when adequate choline is available, despite the considerable differences in the nature of the dietary fat. No differences in the quotient of phospholipin radioactivities and the radioactivities of the total acid soluble phosphorus of the small intestine were found by Zilversmit, Chaikoff & Entenman (1948), despite the feeding of single doses of different fats. In the present experiment, however, the greatest proportion of the weight loss occurred during the first half of the experimental period, and it is therefore possible that changes in the phospholipin turnover rates, due to mobilization and accumulation of fat, might have been detected if measured at some time before the twelfth day.

An increase in phospholipin turnover rates, caused by single doses of choline and high fat diets, has been found by other workers. Perlman & Chaikoff (1939) state that the effect of a single dose of choline is of short duration. In the present experiment when choline was administered it was fed in the diet, and therefore a sharp rise in turnover rate, due to synthesis of new phospholipin molecules, was not to be expected. However, the continuous feeding of choline might have produced a steady state at a higher turnover rate in view of the overall increase in fat metabolism in the palmitic acid fed animals.

The interpretation of the relationship between percentage and amounts of choline containing phospholipins and fat accumulation is difficult. Artom & Fishman (1943b) using 2-3 month old rats, prevented fatty infiltration of the liver by choline supplementation, but did not raise the low values of

choline containing phospholipins observed in the animals on the unsupplemented low protein diet. However, Fishman & Artom (1944), using weanling rats, not only prevented the infiltration of fat by choline supplementation, but raised the percentage and absolute amounts of the choline containing phospholipins, with a corresponding decrease in the non-choline-containing phospholipins. Similar results were obtained by Fishman & Artom (1946) with 100 g male rats. From the present work there is an indication that the amounts of non choline containing phospholipins vary directly with the amount of acetone soluble lipids, while the amount of choline containing phospholipins may be constant as in the palmitic acid fed animals. Channon & Wilkinson (1936) selected natural fats to give a series of iodine numbers and found no correlation between amounts of lecithin and fat accumulation, but unfortunately cephalin was not estimated. Our evidence for the different effects of dietary oleic and palmitic acids on the amounts of liver choline containing phospholipins indicate the importance of considering the nature of the fatty acids in work on phospholipin metabolism.

If the rate of renewal of the fatty acid of the phospholipin differs widely from that of the phosphate, it is possible that tracer studies with marked fats would show that changes in amounts of phospholipins are accompanied by changes in molecular turnover rates. From the preliminary experiment on the molecular rate of turnover of phospholipin phosphorus it was estimated that a change in metabolism of approximately 30 mg of fatty acid by the phospholipins could be detected, on the assumption that fatty acids were renewed at the same rate as the phosphorus. It might have been expected that there would be changes in phospholipin turnover rates related to either the changes in overall fat metabolism or to differences in liver lipid accumulations. The present work indicates no changes in turnover rates of liver phospholipins despite the mobilization of large amounts of depot fat and the greatly increased fat metabolism in the palmitic acid fed animals. The possibility of removal of accumulated liver lipids by a mechanism involving an increase in phospholipin turnover rates was not apparent in the present investigations, since in the only experiment in which enough choline was supplied to palmitic acid fed animals to allow increases in turnover rates there were only slight accumulations of fat in the livers of their litter mates not receiving choline.

The reduction in amounts of total phospholipin on a body weight basis in the palmitic acid fed as compared with the oleic acid fed animals was the only clearly significant change produced in the phospholipins by the nature of the dietary fat.

Low protein, high fat diets did not produce any changes in the molecular or total turnover rates of the liver phospholipins on an absolute or body weight

basis, from the values observed for animals on stock diet. This is in agreement with Bollman & Flock (1946).

The present work indicates that increases in liver fat accumulations caused by feeding saturated as compared with unsaturated fatty acids may be due to increased fat mobilization and metabolism superimposed on a deficiency of lipotropic factors.

SUMMARY

1 An investigation has been made of the amounts of liver lipid accumulating during the feeding of rats with large amounts of pure saturated and unsaturated fatty acids.

2 Low protein high fat diets containing pure oleic and palmitic acids were fed to rats. Analyses were made of carcass and liver lipids, and liver phospholipins. ^{32}P was employed in the measurement of phospholipin turnover rates.

3 The metabolism of large amounts of dietary palmitic acid is accompanied by simultaneous release of large amounts of depot fat. Metabolism of oleic acid does not impose a similar demand on the fat depots.

4 There is a pronounced difference in the percentage of liver fat of different litters of rats when oleic and palmitic acids are fed, in the animals from the litters in which liver fat accumulates, significantly higher percentages of liver fat are obtained for palmitic acid fed than for oleic acid fed animals.

5 Feeding palmitic acid reduces the amount of total liver phospholipin per unit of body weight as compared with animals fed oleic acid or stock diet.

6 There is some evidence that different relationships exist between the choline containing and non-choline containing liver phospholipins when oleic and palmitic acids are fed.

7 A correlation has been found between the amount of liver lipid expressed on a dry weight basis and the amount of radioactivity per unit of liver inorganic phosphate 4 hr after injection of radiophosphate.

8 Phospholipin turnover rates, measured by employing ^{32}P to mark the phosphate component of the molecule, show no correlation with amounts of liver lipids or with gross changes in fat metabolism caused by palmitic acid feeding. This indicates that either phospholipin turnover rates may not be involved, or that the rate of turnover of radioactive phosphate is not a measure of that of the fatty acid on the phospholipin molecule.

9 The factors causing liver lipid accumulations in animals fed diets containing large amounts of saturated fatty acids are discussed.

The authors would like to thank Prof. B. S. Platt for much helpful discussion and advice in the course of this work. The authors also wish to thank Mr R. Goldberg for skilled technical assistance in the construction of part of the Geiger-Müller equipment.

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Renal Function as Affected by Experimental Unilateral Kidney Lesions

2 THE EFFECT OF CYANIDE

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(Received 14 February 1949)

An earlier paper (Nicholson, Selby & Urquhart, 1938) described some of the functional changes occurring when a mild degeneration of the cells of the proximal convoluted tubules is produced by sodium tartrate. One effect is an interference with the selective permeability of the tubular cells such that there is back diffusion of substances to which the walls of the tubules are normally impermeable (e.g. inulin, creatinine, ferrocyanide). Bobey, Longley, Dickes, Price & Hayman (1942) reported a similar effect with uranium nitrate. It was thought possible that any substance which caused tubular degeneration or which interfered with tubular function might also produce changes in the permeability of the cells lining the tubules. To test the latter possibility the effect of cyanide has been studied. In the isolated kidney Starling & Verney (1925) found that cyanide completely inhibits tubular activity and results in the excretion of a urine which is essentially an ultrafiltrate of the plasma, i.e. unchanged glomerular filtrate.

The present experiments show, however, that when cyanide is added to the blood flowing through the kidney *in situ* its nephrotoxic action is more selective.

METHODS

Physiological techniques

Preparation of the animals Female dogs of from 7 to 8 kg were used. Under nembutal anaesthesia the femoral artery and vein on both sides and one jugular vein were exposed. The kidneys were exposed through a long mid line incision, and the renal artery and vein on each side were gently freed from the surrounding perirenal fat. When the cut surfaces had ceased oozing the animal was heparinized. Thin walled silver cannulae, of approximately the same internal diameters as the vessels in which they were to be used, were inserted into the renal and femoral vessels. The femoral and renal veins and the femoral and renal arteries were joined by moderately thick walled rubber tubing running through the abdominal cavity. Each length of rubber tubing had a glass T tube inserted in its course so that one end of each T tube was in immediate juxtaposition with the free end of the respective femoral cannula. The side arms of the arterial T tubes were each attached to a mercury manometer. From each venous T tube a short piece of rubber tubing, closed by a pinchcock, led into a 500 ml cylinder. A graduated reservoir contained 1500 ml of heparinized blood, obtained immediately before the experiment from dogs whose blood gave no cross agglutination with that of the experimental animal, was attached by a rubber tube, closed by a pinchcock to a cannula inserted into the jugular vein. The urinary

bladder was then opened down the mid line anteriorly and the ureters catheterized

Injection of cyanide The rate of blood flow through the kidneys was determined by placing bull dog clamps on the femoral veins just distal to the T tubes and simultaneously opening the pinchcocks on the outlet tubes. The blood was allowed to flow into the graduated cylinders for 5–10 min, depending on the rate of flow, and the minute flow measured. At the same time the dog was transfused through the jugular vein at approximately the same rate as the combined outflow from the kidneys. No appreciable change in renal blood pressure or blood flow was noted during the time of measurement. The rate in different animals varied from 40–75 ml/min and was usually constant throughout the period of measurement. Except in a few animals, which were discarded, the rate of blood flow was practically identical for each kidney of the same animal. When the renal blood flow had been determined a continuous fine stream of 0.07 M NaCN solution of pH 7.4 was introduced into the blood going to the left kidney at the rate of 0.25 ml for every 100 ml of blood. This gives a concentration of cyanide in the blood of approximately 1/600 as used by Starling & Verney (1925). The solution was injected through a no. 27 needle inserted through the wall of the rubber tubing connecting the femoral and renal arteries at a point just proximal to the manometer T tube. The needle was connected to a modified Woodyatt pump by 2 ft of pure gum rubber tubing of $\frac{1}{8}$ in inside diameter and $\frac{1}{16}$ in wall. With this arrangement the solution could be delivered with a steady flow at rates of 0.1–1.0 ml/min. Under these conditions complete mixing of the blood and injected fluid takes place. This was shown by some preliminary experiments in which solutions containing 8% creatinine were injected at the same rate as the cyanide solution. Samples of blood taken from the renal artery contained 20 mg (± 1 mg) of added creatinine/100 ml.

During the period of cyanide injection, which lasted for 13 min, the blood from the renal veins was again run into the graduated cylinders and the blood flow measured, the blood lost being replaced by transfusion from the reservoir. No significant changes in blood flow or blood pressure were produced by the injection of cyanide. The blood from the right kidney was returned at 2 min intervals to the transfusion reservoir. The cyanided blood from the left kidney was discarded. After discontinuing the cyanide injection the blood flow from the right kidney was again directed into the right femoral vein. That from the left kidney was allowed to flow into the cylinder for a further 2 min to wash away any traces of cyanide before directing the flow into the left femoral vein.

Collection of urine and blood Urine was collected under oil in tubes graduated to read to 0.02 ml.

Blood was taken with a tight-fitting oiled syringe from the 'arterial' tubing just distal to the renal artery of each kidney in the first four dogs. As there was no essential difference found between the bloods from the two arteries, samples in the remaining dogs were taken from the right 'arterial' tubing just before it entered the abdominal cavity. The blood (already heparinized in the animal) was centrifuged in a completely filled tube (Peters & Van Slyke, 1931) and all estimations done on the plasma.

Clearance technique Immediately after the animal was anaesthetized a priming infusion of 1 g creatinine, 1.5 g inulin and 25 mg phenol red in 50 ml of 0.89% saline was given into a vein in the fore leg at the rate of 10 ml/min. This was followed by a sustaining infusion of 1 g creatinine,

1 g inulin and 125 mg phenol red in 500 ml 0.89% saline given at the rate of 2 ml/min. This maintained a fairly constant level of the above three substances in the blood for the period during which the clearances were being estimated.

Two consecutive 10 min clearance tests were made before the injection of cyanide. The 'cyanide' clearances were started 3 min after the commencement of the cyanide injection and were continued for 10 min. In a few cases the collection was made in two periods of 5 min each. Blood was collected before and after each clearance period. Clearances of creatinine, inulin, urea and phenol red were estimated for each kidney, and the amount of glucose and chloride in the urine determined. Plasma urea was determined on a specimen taken at about the middle of the experimental period. Plasma creatinine, inulin and phenol red were determined on all specimens. Clearances were

calculated by the usual formula $\frac{U \times V}{P} = C$, where U = con-

centration in the urine in mg/100 ml, V = urine flow in ml/min, P = concentration in the plasma in mg/100 ml and C = amount of plasma 'cleared' in ml/min.

Analytical methods

Plasma Urea was determined by the method of Van Slyke & Cullen (1916).

Inulin was estimated by determining the reduction obtained after hydrolysing a tungstic acid filtrate (Van Slyke & Hawkins, 1928), from which the glucose had been removed by fermentation with washed baker's yeast, with $N H_2SO_4$ for 2 hr at 90°. A blank determination was done on plasma obtained before starting the infusions. Reduction was measured by the Harding & Downs (1933) modification of the Shaffer-Somogyi reagent. Creatinine was determined by the method of Folin & Wu (1919) using the Evelyn photoelectric colorimeter. Total phenol red was determined by adding 5 ml 10% Na_2CO_3 solution to 10 ml of a 1 in 10 dilution of plasma and measuring the colour developed in the Evelyn photoelectric colorimeter using a no. 540 filter.

Free phenol red in plasma was calculated using the formula $\frac{X}{M} = KC$ (Grollman, 1925), where X = mg bound dye/100 ml, M = percentage of albumin, K = 0.66 for dog plasma (Shannon, 1935), C = mg free dye/100 ml and $1/n$ = 0.83 for dog plasma (Shannon, 1935).

Chloride was determined by the titrimetric method of Sendroy (1937), and pH with the Beckman pH meter using the glass electrode.

Urine Urea was estimated as for plasma using 1 in 50 or 1 in 100 dilutions.

Inulin was estimated by the reduction produced on hydrolysis using a 1 in 400 or 1 in 800 dilution of urine. At these dilutions the small amounts of glucose in the urine gave no reduction and the blank was negligible.

Phenol red was estimated as in plasma using dilution of 1 in 400 to 1 in 800.

Glucose was estimated on $HgSO_4$ $BaCO_3$ filtrates (West, Scharles & Peterson 1929) by determining the decrease in reducing values after fermentation with baker's yeast.

Creatinine was determined on 1 in 400 to 1 in 800 dilutions of urine by the method used for plasma.

Chloride was determined by Sendroy's 'exact' titrimetric method and pH with the Beckman pH meter with a glass electrode.

RESULTS

Table 1 gives all the findings in a typical experiment (dog 5). In Table 2, the results obtained on both right and left kidneys during the period in which the blood containing M/600 cyanide was flowing through the left kidney are given.

It will be noted that cyanide has little effect on glucose reabsorption. Urine volume is increased from about 5 to approximately 15 times, but never approaches the volume of the glomerular filtrate. Urine chloride and pH are the same as that of the plasma. The clearances of inulin and creatinine are unaffected, but the clearance of urea is decreased to

Table 1 *Effect of cyanide on renal function*

(Period no. 1 before, period no. 2 during the injection of cyanide into the left renal artery,
R K = right kidney, L K = left kidney)

Period no	Glucose (mg/100 ml)		Chloride (mg/100 ml)			pH			Urine volume (ml/min)	
	Urine		Urine		Plasma	Urine		Plasma	Urine	
	R K	L K	R K	L K		R K	L K		R K	L K
1	9.8	14.1	799	816	371	6.05	6.00	7.47	0.28	0.3
2	16.5	31.8*	773	369*	366	6.10	7.48*	7.46	0.33	3.3*

Period no	Clearances										U/P ratio†		
	Creatinine		Inulin		Urea		Phenol red		'Free' phenol red L.K.	Creatinine		Urea	
	R K	L K	R K	L K	R K	L K	R K.	L.K.		R K	L K	R K	L K.
1	46	44	43	45	30	31	63	60	—	143	150	101	104
2	44	42*	41	43*	31	17*	61	11.3*	45*	150	14*	104	5*

* Blood flowing through left kidney for this period contained m/600 sodium cyanide

† Urine/plasma ratio

Table 2 *Effect of cyanide on certain renal functions*

(Sodium cyanide (M/600) introduced into the blood going to the left kidney
Right kidney served as control)

Dog no	Kidney	Glucose (mg /100 ml)	Chloride (mg /100 ml)		pH		Urine volume (ml /min)	Clearances					'Free' phenol red	U/P ratio*	
			Urine	Plasma	Urine	Plasma		Creat inine	Inulin	Urea	Phenol red	Creat- inine		Urea	
1	R	14.1	867	393	6.13	7.43	0.47	40	39	19	63	—	85	41	
	L	36.5	400	399	7.40	7.42	3.87	38	37	9	8.0	40	9.8	2.3	
2	R	11.7	799	385	5.92	7.51	0.35	52	54	30	85	—	148	86	
	L	28.2	389	390	7.53	7.50	3.15	55	51	16	11.7	49	17.5	5.1	
3	R	19.8	807	397	6.76	7.39	0.93	48	51	28	95	—	52	30	
	L	34.1	396	400	7.43	7.41	4.65	50	49	16	13.3	51	10.8	3.4	
4	R	8.2	826	387	5.57	7.44	0.40	41	39	17	58	—	103	43	
	L	15.6	393	389	7.40	7.42	4.18	38	35	10	8.6	39	9.1	2.5	
5	R	16.5	846	371	6.00	7.47	0.30	44	45	31	60	—	150	103	
	L	31.8	369	366	7.48	7.46	3.30	42	43	17	11.3	45	14	5.2	
6	R	9.4	759	385	6.24	7.48	0.38	54	51	32	87	—	142	84	
	L	21.0	390	384	7.45	7.47	4.51	47	47	17	12.6	48	11.1	3.8	
7	R	8.2	859	383	6.37	7.45	1.13	55	53	22	108	—	49	19.5	
	L	22.3	372	379	7.45	7.46	5.79	55	54	12	14.7	50	9.5	2.1	
8	R	7.0	907	403	6.05	7.47	0.45	50	51	34	94	—	111	76	
	L	20.0	413	409	7.48	7.48	3.97	52	50	18	13.6	56	13.1	4.6	
9	R	10.6	778	382	5.74	7.39	0.42	80	81	46	127	—	191	109	
	L	25.9	377	381	7.40	7.38	4.36	77	80	26	19.6	86	17.7	6.5	
10	R	8.2	921	413	7.92	7.54	0.87	54	56	38	112	—	62	44	
	L	28.2	415	410	7.55	7.53	5.23	50	53	20	16.1	55	9.6	1.8	
11	R	15.7	867	387	7.98	7.53	1.07	53	55	34	83	—	50	47	
	L	20.0	392	389	7.53	7.54	6.43	56	53	19	11.9	54	8.7	1.4	
12	R	9.1	883	389	7.97	7.52	0.40	46	47	30	86	—	115	75	
	L	15.3	395	391	7.50	7.51	5.68	42	44	16	11.7	48	7.4	2.8	

* Urine/plasma ratio

almost one half of the normal value. The 'total' phenol red clearance is only 15 % of normal. When the phenol red clearance is calculated using the figure for 'free' phenol red in the plasma it is found to be practically the same as the creatinine and inulin clearances.

DISCUSSION

The main difference between the effect of cyanide on the isolated kidney as reported by Starling & Verney (1925) and its effect on the kidney *in situ* lies in the continued ability of the latter to reabsorb glucose after poisoning with cyanide and in its ability to concentrate the urine to a considerable extent.

The fact that the clearance of creatinine and inulin is not changed during the period of cyanide action shows that the permeability of the cells to these two substances is unchanged. The marked decrease in urea clearances is presumptive evidence of increased back diffusion of this substance, as it is inconceivable that the glomerulus could become less permeable to urea and still filter inulin and creatinine at the normal rate. This increased back diffusion could be due to a change in cellular permeability for urea. It seems more likely, however, that the cells of the distal tubules and the collecting tubules have to perform work, involving the use of enzyme systems inhibited by cyanide, to prevent urea from diffusing back from the concentrated tubular urine, in contrast to inulin and creatinine which probably remain in the lumen of the tubules because the cell membranes are not permeable to these substances.

The marked diuresis is further evidence that the oxidative systems in the cells are necessary for the performance of much of the osmotic work of the kidney. On the other hand, reabsorption of normal amounts of glucose is little affected and can apparently proceed anaerobically. Whether or not the total ability to handle glucose is decreased was not determined, as we were unable to establish the glucose *T_m* (the maximum amount of glucose which the kidney can reabsorb in 1 min) in the short time in which it was practical to carry on the experiment, but it seems quite probable that this might be the

case in view of the small but consistent increase in glucose in the urine, and the partial decrease in the ability of the kidney to concentrate the urine and to prevent the back diffusion of urea. The secretory powers of the kidney, at least in respect to phenol red excretion and ammonia formation, appear to be concerned with oxidative mechanisms as they are completely abolished by cyanide. The loss of the kidney's ability to change the pH of the urine from that of the glomerular filtrate may be explained on the basis of the mechanism of urinary acidification advanced by Pitts & Alexander (1945), by the suspension of the oxidative metabolic processes within the tubular cells with a consequent lack of carbon dioxide for the formation of carbonic acid, the probable source of the hydrogen ions exchanged for the sodium ions in the tubular urine.

Poisoning of the kidney with cyanide therefore, which inhibits the action of some of the enzyme systems within the cell without producing morphological changes, does not alter the character of the tubular wall as a semi permeable membrane, but does abolish a number of its secretory powers and markedly decreases the amount of osmotic work which can be performed.

SUMMARY

1 A method for perfusing one kidney *in situ* with cyanided blood, while using the other kidney as a control, is described.

2 The general concentrating power of the kidney poisoned with cyanide is only about one tenth that of the normal kidney and its ability to concentrate chloride is entirely lost.

3 Clearances of creatinine and inulin are unchanged, but the clearance of urea is decreased 50 %.

4 The tubular secretion of phenol red and ammonia is completely inhibited.

5 The kidney loses its ability to change the hydrogen ion concentration of the glomerular filtrate.

6 The reabsorption of glucose is practically unaffected.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 277th Meeting of the Biochemical Society was held in the Department of Chemistry, The University, Nottingham, on Friday 17 June 1949, when the following papers were read

COMMUNICATIONS

Separation of the Various Penicillins by Partition Chromatography By F DOBSON and S W STROUD (*Biochemistry Division, Research Department, Messrs Boots Pure Drug Co Ltd, Nottingham*)

Various authors have previously described methods for the separation of the various penicillins based on the silica gel partition chromatogram of Martin & Synge (1941) Catch, Cook & Heilbron (1942) used a modified silica gel chromatogram for the purification of crude penicillin, but this was capable of little development Levi (1948) dealt with the theoretical aspects and the use of a silica gel partition chromatogram, and Boon, Calam, Gudgeon & Levi (1948) described a method for the analysis of crude penicillin using this type of column Fischbach, Eble & Mundell (1947) also described the separation of the various penicillins by means of a silica gel/phosphate buffer partition chromatogram, in which a high degree of separation occurred, but omitted to give the precise details of the method All these methods used silica gel as the absorbent, the silica having to be extremely carefully prepared and standardized to obtain reproducible results (Harris & Wick, 1946)

In the course of the present investigations it has

been found that efficient separation of the various penicillins can be effected by the use of a kieselguhr/sodium citrate buffer partition chromatogram, no pre treatment of the kieselguhr being necessary Optimum conditions were established with 40% (v/v) saturated sodium citrate buffer solution of pH 5.7 as the stationary phase, ether being used as the mobile phase Under these conditions the separated penicillins can be rapidly eluted from the column, and we have been able to prepare 500–1000 mg quantities of the various penicillins in a pure state from 4 to 6 g of commercial samples of mixed penicillins on a 500 g kieselguhr/250 ml sodium citrate buffer column The precise loading on the column is dependent on the relative proportions of the various penicillins in the sample

Small variations in pH have a considerable effect on the efficiency of the separations, and increasing the concentration of the buffer above the established optimum decreases the effective length of the column

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Acetic Acid in Bovine Peripheral Blood and its Utilization by the Mammary Gland By G L McClymont (Walter and Eliza Hall Veterinary Research Fellow, University of Sydney) (introduced by R SCARISBRICK) (*Institute of Animal Pathology, University of Cambridge*)

An arteriovenous difference of volatile fatty acids on the head of the sheep was demonstrated by Reid (1949) Work is now reported on the volatile fatty acid metabolism of bovines with special reference to arteriovenous differences on the mammary gland

Arterial blood was obtained by rectal puncture of the internal iliac (Graham, Kay & McIntosh, 1936), or from carotid loop established in bovines by a

modification of the operation described for goats by Graham, Turner & Gomez (1937) Volatile fatty acid was isolated from arterial blood, by the method of Friedemann (1938) using a metaphosphoric acid filtrate The method of partition chromatography described by Moyle, Baldwin & Scarisbrick (1948) was used for identification and estimation of the acids With diets of grass hay and concentrates, the

volatile fatty acid was found in five cows to be over 91 % (molar basis) acetic, with small proportions of propionic, butyric, and at least two higher acids. For most purposes the volatile fatty acid in bovine peripheral blood can therefore be regarded and calculated as acetic. Reid (1948), using the method of Elsdén (1946), found 100 and 96 % acetic acid in two blood samples from sheep.

Volatile fatty acid, calculated as acetic, was routinely estimated by a modification of the method of McClendon (1944). In three cows studied, acetic acid levels in arterial blood usually reached a maximum within 2–4 hr of feeding, typically attaining levels of 8–12 mg/100 ml of blood, declining to 3–6 mg/100 ml 24 hr after feeding and to 1.5–3 mg/100 ml 48 or more hours after feeding.

Both the non-lactating and lactating mammary gland removed acetic acid from the blood, the arteriovenous difference of the lactating gland in the

fed animal usually being from 40 to 80 % of the arterial level or 2–6 mg/100 ml of blood.

These *in vivo* findings are in accord with the *in vitro* work of Folley & French (1949) on ruminant mammary gland tissue slices.

An association was found between the decline in the Reichert Meissl value of milk fat in starvation, as described by Smith & Dastur (1940), and the fall in the arteriovenous difference of acetic acid, using oxytocin injections to empty the udder as completely as possible at each milking. However, neither intra-ruminal infusion of acetic acid nor intravenous infusion of sodium acetate affected the starvation lowered R M value. This confirms the findings of Mann & Shaw (1947) and Folley & Malpress (cited by Folley & French, 1949), but the explanation suggested by Folley & French, that adequate blood levels of acetate were not obtained, was found invalid for these experiments.

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Microbiological Assay of Growth Factors Essential for *Lactobacillus leichmanni* 313 and *Leuconostoc citrovorum* By K. A. LEES and W. B. EMERY (Fermentation Division, Glaxo Laboratories Ltd., Barnard Castle)

Microbiological tube assays for the determination of Smith's A P A F and other growth factors using *Lactobacillus leichmanni* and *Leuconostoc citrovorum* as test organisms have been developed.

Lac. leichmanni is maintained by daily transfer in liquid medium (yeast extract 1 %, tomato juice 5 %, skimmed milk ad 100 %). *Leu. citrovorum* is maintained in stab cultures (glucose 0.5 %, yeast extract 0.3 %, agar 1.5 %) by fortnightly transfer.

Lac. leichmanni inoculum is prepared by two daily transfers in basal assay medium plus 0.2 % Examene. The second transfer is centrifuged, the cells washed once with saline and resuspended to B W opacity 4. This suspension is diluted 1/8 in a mixture of equal volumes of saline and clarified tomato juice (sterilized by filtration). 0.5 ml of this inoculum is used per tube. *Leu. citrovorum* inoculum is produced similarly from a single 24 hr transfer from the stab culture

into the supplemented basal medium. This is made to opacity 4/8 in saline only.

The basal medium used for both organisms is that of Snell, Kitay & McNutt (1948), modified by omission of enzymatic casein digest and reduction of the level of salts B. To prevent precipitation in the tubes and consequent high blanks, the medium is preheated in bulk and filtered hot. 6 × 1 in. tubes are used, each tube containing 8 ml of medium. Tubes are autoclaved at 15 lb. pressure for 10 min. Incubation is normally carried out at 37° for 16 hr and turbidity measured on a Spekker absorptiometer. Maximum response of *Lac. leichmanni* is obtained with 0.005 µg of Smith's crystalline factor per tube. Maximum response of *Leu. citrovorum* is obtained with 1 ml of 1 mg/ml Examene per tube. Both factors are sterilized by filtration and added aseptically to the tubes.

Leu citrovorum does not respond to 2000 times the level of Smith's factor required to stimulate *Lac leichmanni*

Both factors have been found to be present in refined liver extracts and in concentrates prepared from fermentation residues, shown to be clinically

active in pernicious anaemia. The *Leu citrovorum* factor appears to be more stable to heat than the *Lac leichmanni* factor. Stability tests on Examen at 37° indicate that the *Lac leichmanni* factor is labile at alkaline pH and the *Leu citrovorum* factor is labile at acid pH.

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The Influence of Dietary Protein Levels on Experimental Liver Injury in Dogs By C. J. BARDAWILL and A. G. GORNALL (*Department of Pathological Chemistry, University of Toronto*)

For many years patients with liver disease received diets rich in carbohydrate, but low in protein and fat. About 10 years ago, as a phase in the elucidation of the lipotropic food factors, the value of additional dietary protein was noted and became emphasized. Patek & Post (1941) reported very encouraging results in the treatment of cirrhosis, using a generous diet containing 140 g. of protein, with added B vitamins, and liver extract injections. This work initiated a rapid trend to high protein dietary regimes in liver disease, intakes of 200–300 g. a day being advocated by Morrison (1947) and others. From this one might infer that experimental hepatic damage should tend to be more marked and heal less rapidly in animals kept on a relatively low protein diet.

Carbon tetrachloride has been administered twice weekly by stomach tube to two small groups of adult dogs maintained on isocaloric diets containing approximately 17 and 70 g. of protein per day respectively. After 50–75 doses of the toxin early cirrhotic changes were present in all of the animals, but no distinction could be drawn between the two groups, and it was decided to repeat the experiment on the same animals under more closely comparable

conditions. After a convalescent period the animals were placed again on the different diets, given sixteen consecutive doses of carbon tetrachloride and allowed to recover. Following a further rest period the groups were reversed as to diet and the toxin administration repeated.

The results, measured by the bromsulphalein excretion test, the serum alkaline phosphatase, and the serum albumin and globulin concentrations, showed during and after the first sixteen doses of toxin no appreciable difference between the group on the lower and higher protein intake. Individual responses to the carbon tetrachloride were, however, quite varied. On reversing the two groups it was again impossible to draw any clear distinction between the effects of the toxin. Most notable was the similarity in the response of each individual animal on the two different diets.

The experimental results are regarded as lending support to the view that a large amount of protein (*per se*) is of doubtful benefit in the diet of patients with chronic liver disease. A nutritious diet containing moderate amounts of first class protein (supplemented possibly with certain accessory food factors) may indeed be entirely adequate and perhaps best.

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The Conversion of Acetate to Glycine in the Rat By H. R. V. ARNSTEIN and A. NEUBERGER (*The National Institute for Medical Research, London, N.W. 3*)

Adult rats (Albino Institute strain) were administered sodium acetate labelled with ¹⁴C on the methyl and on the carboxyl groups respectively. The rats were given at the same time DL α -amino γ -phenylbutyric acid and sodium benzoate. Hippuric acid and L α -acetamido γ -phenylbutyric acid were isolated from the urine by counter current ex-

traction with chloroform and ethyl acetate and their radioactivity determined (Table 1).

The activities of the hippuric acid show that both carbon atoms of acetate appear in the glycine molecule, though the specific activity of the hippuric acid is very much smaller than that of the acetamido compound.

Table 1 *All counts were determined with infinite thickness samples (20 mg or more of substance per cm²) on a 1.2 cm² disk. Under these conditions, a substance with a specific activity of 1 μ C/mg gave 10⁵ counts/min/mg*

Rat no	Counts/min/mM					
	L- α Acetamido γ phenylbutyric acid	Hippuric acid	Xanthidryl urea	Diluted hippuric acid	Formaldehyde dimedon	BaCO ₃ from carboxyl group
7*	6.25 \times 10 ⁵	1.325 \times 10 ⁴	4.05 \times 10 ⁴	3.5 \times 10 ³	No activity	3.48 \times 10 ³
8*						
9†	3.0 \times 10 ⁵	1.25 \times 10 ⁴	2.20 \times 10 ⁴	4.0 \times 10 ³	2.45 \times 10 ³	1.18 \times 10 ³
10†	2.25 \times 10 ⁵	2.18 \times 10 ⁴	3.82 \times 10 ⁴			
11§	4.03 \times 10 ⁵	1.51 \times 10 ⁴	Not isolated	1.51 \times 10 ⁴	2.85 \times 10 ³	2.02 \times 10 ³
12§						

* Given 22.6 μ C CH₃C¹⁴O₂Na orally

† Given 18.75 μ C C¹⁴H₅CO₂Na parenterally in three equal doses at two hourly intervals

‡ Given 18.75 μ C C¹⁴H₅CO₂Na parenterally in a single dose

§ Given 18.75 μ C C¹⁴H₅CO₂Na orally

|| This sample was diluted at the glycine stage

The hippuric acid was hydrolysed and the isolated glycine degraded with ninhydrin, the formaldehyde was precipitated as the dimedon derivative and the CO₂ as BaCO₃. The carboxyl carbon atom of acetate is transformed exclusively into the carboxyl carbon of glycine, no activity appearing in the dimedon derivative. With methyl labelled acetate activity was found in both fractions, although the specific activity was always higher in the methylene group (Table 1). These findings are compatible with the interpretation that acetate enters the tricarboxylic

acid cycle, is converted into pyruvic acid and is then transformed into serine by reversal of the reaction studied by Chargaff & Sprinson (1943). The serine is then degraded to glycine (Shemin, 1946). The relatively low activity of the hippuric acid is explained by the fact that most of the glycine used for conjugation with benzoic acid is obtained from the free glycine present in tissues, glutathione and proteins. It is suggested that the synthesis from acetate represents a major pathway for the synthesis of glycine from nitrogen-free precursors.

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Studies on Porphyrin Metabolism in the Rabbit By J. S. F. NIVEN and A. NEUBERGER (*National Institute for Medical Research, London, N.W. 3*)

Adult rabbits were given 31.5 atom % excess ¹⁵N, usually over a period of 1–3 days. Blood samples were collected at intervals and crystalline haemin prepared. Some of the animals also received small doses of sodium benzoate, and hippuric acid was isolated. The results show that the isotope appears in the haemin within a day or less after the administration of glycine has started. On a high protein diet the isotope content of the haemin reached the maximum value (about 0.2%) about one day after the administration of glycine had stopped. The values of the ¹⁵N content of the newly formed haemin and of the hippuric acid excreted during the same period appear to be almost identical in rabbits kept on the high protein diet. On a low protein diet the ¹⁵N content of the haemin rose to almost 0.4% and, under these conditions, the isotope content of the haemin continued to rise for some days after the administration of glycine had been stopped. The ¹⁵N content of the

hippuric acid excreted by rabbits on the low protein diet was markedly lower than the calculated ¹⁵N content of the newly formed haemin, suggesting that under such conditions, the hippuric acid is largely formed from glycine fractions of lower isotope content than those used for the formation of porphyrins.

The life span of the red cell in the normal rabbit was found to vary between 60 and 65 days.

In rabbits made anaemic either by bleeding or by acetyl phenylhydrazine until the haemoglobin content was reduced to about 30%, porphyrin incorporation into the red cells was greatly increased, as shown by the ¹⁵N results. Differential centrifugation showed that the ¹⁵N content in the reticulocytes was particularly high. However, most of the cells formed in the recovery period appear to have a much shorter life span than cells formed under normal conditions.

Effects of Electrolytes on the Molecular Weight of Tropomyosin By G S ADAIR, K BAILEY and T C TSAO (*Biochemical Laboratory, and Low Temperature Research Station, University of Cambridge*)

The molecular weight of tropomyosin (TM) from rabbit skeletal muscle dissolved in a salt solution was found to be 90,500 by the methods of osmotic pressure, sedimentation-diffusion and amino acid analysis (Bailey, Gutfreund & Ogston, 1948). In salt free solutions TM aggregates into large fibrils (Bailey, 1948, Astbury, Reed & Spark, 1948) which disaggregate again on addition of salt. In order to determine the effects of salts on the degree of aggregation of tropomyosin, the mol wt of TM has therefore been reinvestigated by osmotic pressure measurements, employing the toluene and tetra chloroethylene osmometers of Adair (unpublished), and for the calculation of results the extrapolation procedure of Adair & Robinson (1930). In Table 1 are listed the values obtained (a) in neutral salt solutions of varying ionic strength, (b) in concentrated urea solutions, and (c) in acid solution (pH 2).

molecular weight deduced from the histidine content, viz $3 \times 18,180 = 54,540$. The higher molecular weights in the other series must be considered average values for mixtures of single, double and multi molecular species. With the exception of protein recovered from urea solutions the TM in all series could be crystallized.

Previous work with the ultracentrifuge (Bailey, Gutfreund & Ogston, 1948) has shown that solutions of TM which are now considered to contain several molecular species in varying states of aggregation give a single sedimenting boundary. This behaviour seems characteristic of systems containing markedly asymmetric particles (Campbell & Johnson, 1944).

Another interesting feature is the high degree of osmotic pressure concentration dependence. This can be seen from the values of ϕ in Table 1, where ϕ is a coefficient representing the effects of all factors

Table 1 Particle weights of tropomyosin

($C_p = g$ protein/100 ml. solvent)

Buffer	pH	Ionic strength	Particle wt	ϕ	
				$C_p = 1$	$C_p = 3$
Phosphate (0.06 M)	6.5	0.10	135,000	1.40	2.60
Phosphate (0.06 M) + NaCl (0.1 M)	6.5	0.20	112,000	1.43	2.88
Phosphate (0.04 M) + KCl (0.2 M)	6.5	0.267	88,000*	1.14	1.44
Phosphate (0.06 M) + NaCl (0.2 M)	6.5	0.30	72,000	1.17	1.51
Phosphate (0.06 M) + NaCl (0.5 M)	6.5	0.60	67,000	1.22	1.66
Phosphate (0.03 M) + NaCl (1.05 M)	6.5	1.10	65,000	1.21	1.62
Phosphate (0.075 M) + urea (6.67 M)	6.5	0.1	53,000	1.73	3.24
KCl (0.09 M) + HCl (0.01 M)	2.1	0.1	51,000	1.37	2.84

* Data of Bailey *et al.* (1948)

In phosphate buffers (pH 6.5) the mol wt falls exponentially with increase of ionic strength, approaching asymptotically the values of 51,000 and 53,000 obtained in acid and urea respectively. The mean value conforms with that based on the minimal

causing deviation from the simple form of van't Hoff's law. Even in buffers of high salt concentration, where the Donnan effect is small, the values of ϕ are still high compared with those of other proteins (cf Adair & Robinson, 1930).

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Separation of *N*-2 4-Dinitrophenyl Derivatives of the Methyl Esters of Amino-acids by Adsorption Chromatography By A G LOWTHER and W S REITH (REICH) (*Department of Organic Chemistry, The University, Leeds 2*)

N-2 4-Dinitrophenyl derivatives of amino acids were first prepared by Abderhalden & Blumberg (1910) Sanger (1945) applied partition chromatography to the separation of mixtures of these coloured derivatives

It has been found previously (Reich, 1948) that mixtures of the methyl esters of *N* azobenzene *p*-sulphonyl derivatives of some amino acids can be separated quantitatively by adsorption chromatography on alumina columns. We have now prepared the methyl esters of the *N* 2 4 dinitrophenyl derivatives of certain amino acids and find that they can be selectively adsorbed on suitably prepared alumina, a mixture of such derivatives can be resolved by the development of the chromatogram, and, after elution, the unchanged methyl esters can be quantitatively recovered and identified

The alumina for adsorption was prepared by heating aluminium oxide with a 10% solution of acetic acid in methanol, washing thoroughly with methanol and drying, first at room temperature and then at 40° for 24 hr

A mixture of 37 mg of *N* 2 4 dinitrophenyl glycine methyl ester (m p 124°) and 43 mg of *N* 2 4 dinitrophenyl DL alanine methyl ester (m p 121°)

dissolved in benzene (8 ml) was adsorbed on a column formed from a slurry of alumina (130 g) and a solution of 1 vol of benzene in 3 vol of ligroin (b p 60–80°) (250 ml). When all the solution of the esters had been added to the column the chromatogram was developed with 400 ml of the benzene/ligroin solution. Finally each band was removed, and the adsorbed compounds eluted with a mixture of acetone/methanol (1:1)

After evaporation of the acetone/methanol *in vacuo*, the residue was dissolved in 20 ml of benzene, the solution filtered through a glass filter (G 4), and the benzene evaporated *in vacuo*

The appearance of the final chromatogram, and the weights of the recovered compounds were as follows

13 mm colourless	} 34.8 mg <i>N</i> 2 4 dinitrophenyl glycine methyl ester, m p 124–125°
34 mm yellow	
13 mm colourless	
13 mm pale yellow	} 42.7 mg <i>N</i> 2 4 dinitrophenyl- DL alanine methyl ester, m p 121–122°
23 mm yellow	

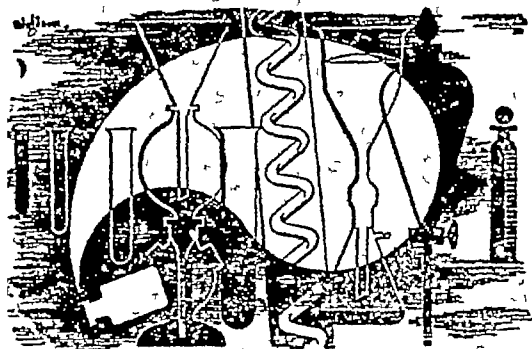
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FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

- The intermediary metabolism of the mammary gland 1 Respiration of lactating mammary gland slices in presence of carbohydrates By S J FOLLEY and T H FRENCH
- Observation on the mono amine oxidase activity of placenta and uterus By R H S THOMPSON and A TICKNER
- Effect of sulphhydryl combining compounds on the activity of the succinic oxidase system By E C SLATER
- The relationship between the constitution and the effect of chemical compounds on plant growth 1 2 Phenoxy-ethylamine derivatives By R L JONES, T P METCALFE and W A SEXTON
- The reduction of gastric acidity by back diffusion of H^+ ions through the mucosa By C TERNER
- Studies in detoxication 26 The fates of phenol, phenylsulphuric acid and phenylglucuronide in the rabbit, in relation to the metabolism of benzene By G A GARTON and R T WILLIAMS
- The biogenesis of porphyrins The distribution of ^{15}N in the ring system By HELEN M. MUIR and A. NEUBERGER
- Factors affecting synthesis of ascorbic acid in cress seedlings 2 Ascorbic acid synthesis in relation to sugar formation By L W MAPSON, ETHEL M. CRUICKSHANK and YU TUAN CHEN
- Studies on metabolic products of the *Penicillium luteum* series By G G FREEMAN and CATHERINE S MACPHERSON
- The sedimentation and diffusion of polysaccharides from *Penicillium luteum* Interpretation of the results obtained from polydisperse material in the Gouy diffusimeter By A G OGSTON
- Metabolic products of *Trichothecium roseum* Link. By G G FREEMAN, R I MORRISON and S E MICHAEL
- Acid soluble pigments of molluscan shells
- 2 Pigments other than porphyrins By A COMFORT
 - 3 The indigoid character of the blue pigment of *Haliotis cracherodii* Leach By A COMFORT
 4. Identification of shell porphyrins with particular reference to conchoporphyrin By R E H NICHOLAS and A. COMFORT
- Distribution and nutritive value of the nitrogenous substances in the potato By H CHICK and E B SLACK
- The pectic enzymes of the mould *Byssoschlamys fulva* By G H BEAVEN and F BROWN
- Studies on suramin (Antrypol Bayer 205) 7 Further observations on the combination of the drug with proteins By E JOAN WILSON and A. WORMALL
- The phosphorus compounds of wheat starch By H. S ROOKE, L H LAMPITT and E M. JACKSON
- Studies in vitamin A 9 The role of the thyroid in carotene and vitamin A metabolism By H R CAMA and T W GOODWIN
- Metabolism of derivatives of toluene 3 *o*, *m* and *p* xylenes By H G BRAY, BRENDA G HUMPHRIS and W V THORPE



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